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Quantifying toxicological stress in amphibians: The influence of hydrophobicity on PAH and PCB elimination rates in northern leopard frogs (Rana pipens).

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Quantifying Toxicological Stress in Amphibians: The Influence of Hydrophobicity on PAH and PCB Elimination Rates in Northern Leopard Frogs (*Rana pipiens*)

By

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Abstract

An experiment was conducted to determine if the elimination rates of various PCB and PAH congeners were controlled by hydrophobicity (log K_{ow}) in adult northern leopard frogs (*Rana pipiens*). Frogs were subjected to intraperitoneal injections of one of the following three treatments: a) 1:1:1 mixture of Aroclors 1242, 1254 and 1260; b) an equimolar mixture of 17 unsubstituted PAHs; c) a sunflower oil blank (control). Animals were sacrificed 1, 2, 4, 8, 16, 24 days after injection, followed by homogenization, chemical extraction and clean-up, and gas chromatographic analyses for remaining parent analytes.

The elimination response of PCB-treated frogs was significantly different from the elimination response of PAH-treated frogs over time (MANOVA $p<0.05$). Linear regression analyses did not detect a significant change in concentration over time in any of the PCB congeners used in the analyses. Linear regression analyses on PAH-treated frogs detected significant ($p<0.05$) and marginally significant ($0.1>p>0.05$) declines over time in several of the PAHs analysed. Elimination rates of these PAHs were as follows: fluorene (0.044 d^{-1}), phenanthrene (0.045 d^{-1}), anthracene (0.038 d^{-1}), fluoranthene (0.027 d^{-1}), pyrene (0.032 d^{-1}), benz(*a*)antrhacene (0.07 d^{-1}), chrysene / triphenylene (0.044 d^{-1}) and benz(*g,h,i*)perylene (0.066 d^{-1}). Regression of PAH elimination rates against hydrophobicity failed to detect a significant linear relationship ($p>0.05$).

Elimination of PAH analytes were generally faster than that of PCBs. Comparison of PAH elimination rates to PCBs of corresponding log K_{ow} gives indirect evidence of biotransformation (metabolism). The persistence and concomitant potential of PCB trophic transmission and narcosis, and the probable biotransformation of some PAHs into genotoxic metabolites could be related to amphibian population declines. The use of adult amphibians for PCB and PAH biomonitoring is also discussed. In addition, the first order, one compartment kinetic model commonly used in aquatic toxicology does not seem to apply to adult amphibians. Because adult amphibians are intermediate between aquatic and terrestrial, kinetic models need to be developed that more accurately reflect the resultant unique physiology.

Dedication

This thesis is dedicated to those who are unable to pursue their educational goals, whose knowledge, intelligence and experience extend far beyond the limits of formal training and recognition.

"Pay no attention to the man behind the curtain"

In "The Wizard of Oz", this was spoken as Toto drew open the curtain, unveiling the real "Great Oz". This simple act was a part of a chain of events that led Dorothy and her friends to the realization that happiness, sympathy, courage and knowledge lies within.

Acknowledgments

This project could not have been completed by myself. There are a number of people across two organizations whose participation and contribution were critical to the completion of this thesis.

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Canadian Forest Service – Great Lakes Forestry Centre

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List of Abbreviations

f	fugacity
C	chemical concentration
Z	capacity of an environmental phase to hold a chemical
K_{ow}	octanol – water partition coefficient
t	time
k_1	rate of chemical uptake from water
k_2	rate of chemical elimination from an organism into water
BCF	bioconcentration factor
TSS	time to steady state
BHL	biological half life
QSAR	quantitative structure-activity relationship
PAH	polycyclic (polynuclear) aromatic hydrocarbon
HAH	halogenated aromatic hydrocarbon
PCB	polychlorinated biphenyl
Ah	aryl hydrocarbon (receptor)
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
MFO	mixed function oxidase
GC	gas chromatograph
ECD	electron capture detector
MSD	mass selective detector
MANOVA	multivariate analysis of variance

Introduction

The scientific community has expressed concern over the apparent global decline of amphibian populations, and many diverse causes of these declines have been postulated (Barinaga 1990; Wyman 1990; Wake 1991; Gannon 1997). Such theories include habitat degradation, climate change, ultraviolet radiation, disease, pollution and combinations of factors (Harfenist et al. 1989; Pounds and Crump 1994; Carey and Bryant 1995; Delis et al. 1996; Blaustein et al. 1997). Physiology and life history characteristics render the amphibian fauna particularly susceptible to environmental degradation and the effects of pollutant chemicals (Roth 1973; Dunson et al. 1992). This susceptibility, along with their central role in aquatic and terrestrial food webs may make amphibians ideal biomonitors of pollutant chemicals (Niethammer et al. 1984; Barinaga 1990).

One class of pollutant chemicals of environmental concern are the polychlorinated biphenyls (PCBs). They are a group of man-made compounds created and used for their heat resistance and electrical insulative properties (Hutzinger et al. 1974). However, the high hydrophobicity and stability that made this class of compounds commercially successful also renders them biologically persistent and resistant to biodegradation (Tanabe et al. 1987). In addition to their potential to cause narcotic effects in an organism through sufficient bioaccumulation and recalcitrance to biodegradation, certain PCB congeners structurally resemble 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Such compounds bind to the aryl hydrocarbon (Ah) receptor, causing the induction of the Mixed Function Oxidase (MFO) enzyme system, whose role is to transform hydrophobic organic molecules into metabolites that are less hydrophobic and easier to eliminate (Newstead et al. 1995; Landers and Bunce 1991). However, metabolism of otherwise nontoxic foreign compounds, such as polycyclic aromatic hydrocarbons, yields metabolites that are known to be genotoxic (Figure 1) (Landers and Bunce 1991).

Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds generated by natural synthetic and pyrolytic events, such as forest fires and the burning of fossil fuels. They are of ecological and toxicological concern because of their environmental ubiquity and potential toxicity (Neff 1979). In addition to their hydrophobicity and concomitant

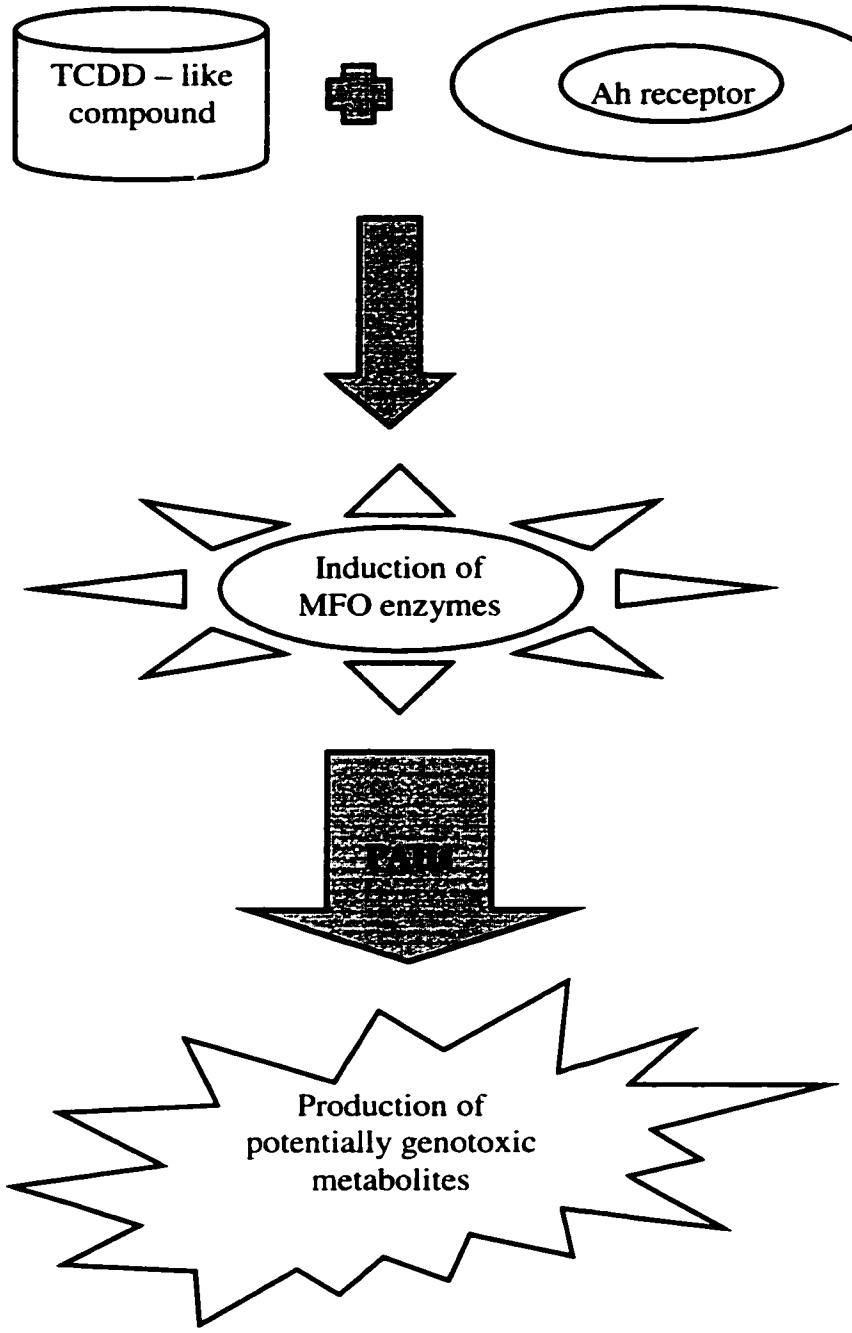


Figure 1. Enzymatic chain of events. TCDD and similar compounds bind to the Ah receptor, which causes the induction of mixed function oxidase (MFO) enzymes. When PAHs are metabolized by these enzymes, the resultant metabolites may be genotoxic.

ability to cause narcosis through sufficient bioaccumulation within an organism, PAHs are susceptible to metabolic transformation by the MFO enzyme system. Some of the resultant metabolites are known to be genotoxic (Foureman 1989).

Adult amphibians have high assimilation efficiencies and considerable capacity to accumulate chemicals (Gillan et al. 1998). The recent detection of PAH and organochlorine residues in adult frogs of southern Ontario (Russell et al. 1995; Russell et al. 1997a; Gillan et al. 1998), and the detection of DNA damage in local northern leopard frog and green frog (*Rana clamitans*) tadpole erythrocytes (Ralph et al. 1996; Ralph and Petras 1997), coupled with the detection of the Ah receptor (Marty et al. 1989) and the characterization and induction of the cytochrome P450-dependent MFO system in amphibians (Noshiro and Omura 1984; Marty et al. 1992; Huang et al. 1998) indicates a potential genotoxic threat to local amphibian communities. Because of the limited dispersal abilities, site fidelity, and metapopulation dynamics of frogs (Hecnar and M'Closkey 1996), exposure to and metabolism of a variety of pollutant compounds could cause genetic stress that may affect the fitness of amphibian communities.

Thesis Objectives

To evaluate the use of northern leopard frogs as chemical biomonitors of PCBs and PAHs, and the potential of these classes of chemicals to cause a narcotic or genotoxic threat, I attempted to:

1. Determine if the elimination of a) PCB or b) PAH congeners are explained by the passive process of simple partitioning based on hydrophobicity (log K_{ow}) in northern leopard frogs (*Rana pipiens*) by examining the relationship between congener elimination rates and log K_{ow};
2. Compare the elimination rate-log K_{ow} relationships for PCBs and PAHs in northern leopard frogs, and evaluate any differences in order to deduce which chemicals are being metabolized.

In this study, northern leopard frogs were used because they satisfy many of the criteria for biomonitoring: they are widely distributed across North America, are locally indigenous, and they are conspicuous and ecologically important (Conant and Collins

1991; Hecnar and M'Closkey 1996). They were also chosen for this study because of their resilience to human handling and intraperitoneal injections compared to other locally available adult frogs species, and their sensitivity to TCDD and similar compounds (Jung and Walker 1997). To the author's knowledge, no other PCB or PAH elimination studies have been conducted on adult anuran amphibians.

Hypotheses

1a) It was hypothesized that for PCBs, elimination rate will decrease with increasing hydrophobicity (K_{ow}). Previous studies show similar trends in a variety of organisms. In earthworms (*Eisenia andrei*), PCB elimination rate constants decreased with increasing K_{ow} (Belfroid et al. 1995). In rainbow trout (*Oncorhynchus mykiss*), PCB bioconcentration factors (BCF) were positively correlated with log K_{ow} (Neely et al. 1974) and influenced by log K_{ow} (Coristine et al. 1996). In zebra mussels (*Dreissena polymorpha*), hydrophobicity was the primary factor influencing PCB elimination (Morrison et al. 1995), and BCFs were positively correlated with PCB and PAH K_{ow}s (Bruner et al. 1994). In green-lipped mussels (*Perna viridis*), lower-chlorinated, less hydrophobic PCBs were taken up and eliminated more rapidly than highly chlorinated, more hydrophobic PCBs (Tanabe et al. 1987). In blue mussels (Mytilus edulis), log BCFs of PCBs showed a positive linear relationship with log K_{ow} (Bergen et al. 1993). In the mussel *Mytilus galloprovincialis*, BCFs of PCBs were positively correlated with log K_{ow} (Porte and Albaiges 1993). In the American oyster (*Crassostrea virginica*), depuration rates of high molecular weight PCBs were significantly lower than with low molecular weight PCBs (Sericano et al. 1996). In early life stages of zebrafish (*Brachydonio rerio*), cod (*Gadus morhua*), herring (*Clupea harengus*) and turbot (*Scophthalmus maximus*), PAH and PCB elimination rates and log K_{ow} were negatively correlated (Petersen and Kristensen 1998). The bioconcentration factors of PCBs were positively correlated with log K_{ow} in the zebrafish (Fox et al. 1994).

1b) For PAHs, however, it was hypothesized that in northern leopard frogs, hydrophobicity will not influence elimination because of the likelihood of metabolism. Numerous studies have shown that PAHs are metabolized in a wide variety of organisms,

and that PAH elimination cannot be solely explained by passive processes such as simple partitioning based on K_{ow}; chemicals that are readily biotransformed will not behave according to predictions based on partition coefficients (Spacie et al. 1983).

The ability of marine invertebrates to metabolize PAHs varies widely, but is generally much less than rates observed in vertebrates (Livingstone 1994). PAH biotransformation in the oligochaete *Stylodrilus heringianus* and scud *Pontoporeia hoyi* was found to be negligible (Frank et al. 1986; Landrum 1982). The blue crab (*Callinectes sapidus*) was able to extensively metabolize PAHs (Lee et al. 1976). Fish are generally able to metabolize PAHs (Varanasi et al. 1989a). Polar metabolites of PAHs have been detected in the bile of brown bullheads from the Buffalo River (*Ameiurus nebulosus*) (Maccubbin et al. 1988). The newt *Pleurodeles waltl*, as well as the sea bass *Dicentrarchus labrax* have been shown to metabolize the PAH benz(*a*)pyrene (Marty et al. 1989; Lemaire et al. 1992). Experimental data from Ma et al. (1998) suggests that earthworms (*Lumbricus rubellus*) have some capacity of PAH metabolism. Bluegill sunfish (*Lepomis macrochirus*) were able to metabolize the PAHs anthracene and benz(*a*)pyrene (Spacie et al. 1983). The terrestrial isopod *Porcellio scaber* may be able to metabolize PAHs, as they are able to eliminate benz(*a*)pyrene quickly (van Brummelen and van Straalen 1996). *Chironomus riparius* was able to biotransform benz(*a*)pyrene (Leversee et al. 1982). *Hyalella azteca* was able to biotransform anthracene to a much greater extent than *Pontoporeia hoyi*, but not as much as *Chironomus riparius* (Landrum and Scavia 1983). The polychaete worms *Nereis diversicolor* and *Scolecolepides viridis* were able to extensively metabolize benz(*a*)pyrene (Driscoll and McElroy 1996). In a seston-blue mussel (*Mytilus edulis*)-eider duck (*Somateria mollissima*) food chain, PAH levels decreased with increasing trophic level, likely due to increasing metabolic activity with increasing trophic level (Broman et al. 1990). The detection of the Ah receptor, as well as the induction and characterization of PAH-metabolizing enzymes in frog livers supports the hypothesis that in adult frogs, metabolism will affect PAH elimination rates more so than hydrophobicity (Marty et al. 1989; Marty et al. 1992; Noshiro and Omura 1984; Huang et al. 1998).

2) It was hypothesized that there will be a difference between the elimination rate – log K_{ow} relationships for PCBs and PAHs in northern leopard frogs. This difference will

likely be caused by metabolism contributing to the elimination of PAHs above and beyond passive partitioning processes based on hydrophobicity. In a study conducted by Ma et al. (1998), elimination rate constants of PAHs failed to show a decline with increasing hydrophobicity, whereas chlorinated benzenes of similar hydrophobicity had the tendency to decrease with increasing hydrophobicity. Metabolism may be the principal factor that can account for differences in bioaccumulation between two compounds with minimal differences in hydrophobicity and steric configuration (Tulp and Hutzinger 1978). In fish, bioaccumulation of high molecular weight hydrophobic compounds that can be metabolized (such as PAHs) tend to correlate poorly with K_{ow}, and such compounds show less bioaccumulation than PCBs (Oliver and Niimi 1985; Van der Oost et al. 1991). In *Chironomus tentans* larvae, metabolism may be responsible for the lower bioaccumulation factors of PAHs compared to that of PCBs (Wood et al. 1997). Comparison of the biological tenacity of persistent compounds (such as PCBs) to that of PAHs may indicate if PAHs are being metabolized, even if metabolites are not measured directly (McElroy et al. 1989).

Scope

The elimination rates of PCB and PAH congeners provide much information regarding their persistence in adult northern leopard frogs, such as biological half-life. Quantifying a relationship between PCB or PAH elimination rates and hydrophobicity (log K_{ow}) will be useful in estimating a PCB or PAH congener's persistence in the northern leopard frog relative to that congener's hydrophobicity. If PCB and/or PAH congeners are persistent in these organisms, northern leopard frogs may be used as biomonitors of these contaminants. The persistence of a contaminant in an organism determines whether or not the organism is suitable to monitor for the presence of a particular contaminant in its environment, or even track changing environmental contaminant concentrations through time. A contaminant's persistence in an organism also gives an indication of the likelihood of trophic transfer, as well as the possibility of the contaminant accumulating within an organism to the point where adverse physiological effects occur (narcosis).

Background

Amphibians in Decline

Recent global declines in amphibian populations are alarming herpetologists (Barinaga 1990; Wyman 1990; Wake 1991). However, due to the lack of long-term amphibian population studies and data (Blaustein et al. 1994; Wyman 1990), there may be problems differentiating between natural amphibian population fluctuations and declines due to anthropogenic causes (Pechmann et al. 1991; Pechmann and Wilbur 1994). Nonetheless, several possible reasons have been postulated for the declines.

Urban development, causing habitat degradation, destruction, and the loss of amphibian breeding sites can cause local amphibian declines (Delis et al. 1996). Climate changes affecting temperature and moisture have caused declines in amphibian species living in relatively restricted, specialized habitats (Pounds and Crump 1994). The depletion of the ozone layer has increased the amount of UV-B radiation reaching the earth's surface; UV-B radiation has been shown to cause species-specific embryonic abnormalities and mortality (Licht and Grant 1997; Blaustein et al. 1997). Diseases and infections, such as redleg (*Aeromonas hydrophila*), could cause mass mortality in restricted populations (Nyman 1986). Recently, a trematode of the genus *Ribeiroia* has been shown to cause limb abnormalities in Pacific treefrogs (*Hyla regilla*), which adversely affects development and survivorship (Johnson et al. 1999). Industrialization and the use of various anthropogenic chemicals are also known to have adverse effects on amphibians and amphibian populations. Contaminated industrial areas in the eastern Ukraine have anuran amphibian (frog and toad) populations with high incidences of developmental abnormalities (Flax and Borkin 1997). The application of ammonium nitrate fertilizer has been shown to have a toxic effect on frogs (Oldham et al. 1997; Hecnar 1995; Berger 1989). The use of a wide variety of pesticides of various chemical classes has been shown to have adverse effects on amphibians, ranging from mortality to sublethal effects such as impaired swimming and hyperactivity (reviewed in Harfenist et al. 1989), as well as developmental abnormalities (Hayes et al. 1997; Ouellet et al. 1997; Osborn et al. 1981).

Combinations of factors may also cause amphibian declines. Kieseker and Blaustein (1995) found a synergism between UV-B radiation and a fungal infection

(*Saprolegnia*) which magnified amphibian embryo mortality in nature. Ankley et al. (1998) and Zaga et al. (1998) found that the insecticides methoprene and carbamate, respectively, were more toxic to larval amphibians in the presence of ultraviolet light wavelengths found in natural sunlight. Similarly, Hatch and Burton (1998) and Walker et al. (1998) found that fluoranthene was more toxic to larval amphibians in the presence of sunlight.

Amphibians may be particularly susceptible to anthropogenic chemicals for a variety of reasons. Their complex life cycles, which include aquatic and terrestrial life stages, expose them to potential chemical contamination in both habitats. Their eggs, gills and skin are permeable to water and electrolytes (Duellman and Trueb 1986). Their highly vascularized skin serves a respiratory function (Duellman and Trueb 1986; Roth 1973), and is in close contact with potentially contaminated sediments, water, and soil, which explains why frogs may be especially sensitive to soil highly contaminated with insecticide (Lambert 1997). Due to the ectothermic nature of amphibians, their metabolic rates are temperature-dependent; during the cold winter months, reduced ability to metabolize contaminant residues could result in their accumulation within an amphibian. Similarly, fossorial habits, aestivation or hibernation in soil or mud could expose them to toxic conditions to which they may be unable to respond (Dunson et al. 1992). As many adult amphibians are insectivorous, they may be exposed to anthropogenic chemicals, particularly insecticides, through contaminated prey (Dunson et al. 1992). Adult frogs have been shown to have a considerable capacity to accumulate contaminants, and have high assimilation efficiencies (Gillan et al. 1998).

Amphibians as Biomonitors

Biomonitoring is the regular or systematic use of living organisms to evaluate changes in environmental quality (Cairns and van der Schalie 1980). Organisms chosen to be contaminant biomonitors should be widely available and abundant, indigenous to or representative of the ecosystem being studied, ecologically important, and be able to accumulate measurable quantities of contaminants in their tissues (Rand et al. 1995). The amphibian fauna may be an important monitor of the impact of anthropogenic disturbance to wetland ecosystems (Hecnar and M'Closkey 1996). In addition to their

susceptibility to environmental contaminants due to their life history characteristics, physiology and their capacity to assimilate contaminants, amphibians may be ideal contaminant biomonitors because they are ecologically important, occupying roles as both predator and prey (Niethammer et al. 1984). They are also indigenous to, widespread and abundant across Southern Ontario (Oldham 1988), and they are visually and vocally conspicuous, especially during breeding, which would facilitate population monitoring (Dunson et al. 1992).

The use of contaminant concentrations in wildlife tissue as an indicator of environmental contaminant concentrations is based on the assumption that there is a fairly constant ratio of tissue to environmental concentration for a particular contaminant (Clark et al. 1988). In order to use a specific organism as a biomonitor, the toxicokinetics (rate of uptake, elimination, metabolism) of a particular contaminant within that organism must be understood, in addition to a thorough knowledge of the fundamental biology, ecology and natural history of the indicator fauna (Clark et al. 1988).

Man has introduced chemicals into the environment both deliberately and as products and by-products of industrial processes. For instance, the open burning of scrap tires creates 3611 kg of polycyclic aromatic hydrocarbons (PAH) for every 1000 tons of tire burned; on-road vehicles emit 20.05μg PAH for every mile traveled (EPA 1998). The extensive use of organic chemicals in industrial and domestic products and applications has resulted in an increase of direct and indirect chemical discharge into the environment. Once a chemical is released into the environment, what happens to it, and where does it go?

The Environmental Fate of Organic Contaminants

Non-polar, organic chemicals dissolve poorly in polar solvents such as water. In the environment, non-polar, organic contaminants tend to go into solution with or be adsorbed to other organic compounds, such as lipid or particulate organic carbon. This passive process, known as partitioning, has chemicals moving into phases (air, water, sediment, biota) in which they can achieve maximum thermodynamic and entropic stability (Mackay 1991). An important part of assessing the environmental fate of

contaminants is predicting the extent to which these substances will concentrate in an organism, which could be much greater than the chemical concentrations in the organism's surroundings (Mackay 1982).

Predicting and quantifying the movement of chemicals in the environment can be approached in two different ways:

1. Equilibrium (Fugacity) Approach

The equilibrium approach describes the mechanisms of the movement of a chemical based on transport parameters and capacities of environmental phases to hold that particular chemical. Fugacity is the tendency of a chemical to partition out of a phase, and is expressed as the pressure exerted by the chemical in relation to its tendency to leave a particular phase (Mackay 1991). Fugacity is based on the thermodynamic assumption that at equilibrium, all environmental compartments or phases will have equal fugacities. The concentrations of a chemical in the different phases will be different, but the tendency for the chemical to leave a phase will be the same. Fugacity can be expressed as:

$$f = C / Z \qquad (1)$$

where f represents fugacity, C is the chemical concentration and Z is the capacity of a phase to hold the chemical (Mackay 1991). Thus, the fugacity of a chemical in water is:

$$f_w = C_w / Z_w \qquad (2)$$

where C_w is the chemical concentration in water and Z_w is the capacity of water to hold that particular chemical. The fugacity of a hydrophobic chemical in an animal can be represented by:

$$f_l = C_l / Z_l \qquad (3)$$

where the lipid phase is the primary storage site of the chemical in the animal.

Because of its availability in pure form, its sparing solubility in water and similar carbon to oxygen ratio, 1-octanol is used as a surrogate for lipids (Mackay 1991). The octanol-water partition coefficient (K_{ow}) represents the ratio of equilibrium concentrations of a chemical in 1-octanol (lipid) and water, and is frequently used as a measure of a chemical's hydrophobicity (Mackay 1991). K_{ow} is expressed as the ratio of the chemical's capacity in 1-octanol (lipid) to the chemical's capacity in water:

$$K_{ow} = Z_o / Z_w = Z_l / Z_w \qquad (4)$$

(Mackay 1991). Although the equilibrium model predicts the direction in which a chemical will move in order to achieve equilibrium, this equilibrium can take a long time to achieve. Fugacity models predict that all organisms will have the same concentration of a chemical per unit lipid (LeBlanc 1995). However, ecosystems are not static: natural processes such as growth, and variable chemical inputs change chemical fugacities, rendering the achievement of chemical equilibrium a rare event. This approach does not predict the rate of chemical movement between phases. The kinetic approach, however, picks up where the fugacity model falls short.

2. Kinetic Approach

The kinetic model predicts the non-equilibrium partitioning of organic chemicals, as well as the rates of chemical movement between phases (Landrum et al. 1992). For the purposes of this study, the kinetic approach will use a one compartment model with first order rate constants for greater comparability with other studies. In a one compartment model, the animal is regarded as a single compartment, and chemicals move into and out of that compartment. The one compartment model is most frequently used in aquatic toxicology (Barron et al. 1990). First order rate constants describe the rate of transfer of a chemical to or from a compartment as being proportional to the concentration of chemical within that compartment. First order rate constants are typically expressed as an amount of chemical being transferred per unit time (Barron et al. 1990). If an organism is placed in a contaminated environment, it will accumulate contaminants until the rate of uptake equals the rate of elimination (steady state). If

chemical uptake is greater than elimination, bioaccumulation takes place. An animal can accumulate contaminants through water, air, food and sediment, with each uptake medium having a unique uptake rate constant. Similarly, an organism can eliminate contaminants through defecation, excretion, volatilization, egg-laying, and other routes, each having a unique elimination rate constant. For the purposes of this study, the term "elimination" will be used to describe the summation of metabolic, excretory and other processes used to discharge a chemical from an organism (Barron et al. 1990).

In general, if an aquatic organism considered to be a single compartment with first order kinetics is placed in water contaminated with a non-biodegradable hydrophobic contaminant, the organism will bioaccumulate the contaminant according to the following equation:

$$dC_a / dt = k_1 C_w - k_2 C_a \qquad (5)$$

where:

C_a represents the contaminant concentration in the organism;
C_w represents the contaminant concentration in the water;
k_1 represents the rate of chemical uptake from water;
k_2 represents the rate of chemical elimination from the organism into water.

(Hawker and Connell 1985). The bioconcentration factor (BCF) is the ratio between uptake and elimination rates to and from water:

$$BCF = k_1 / k_2 \qquad (6)$$

(Hawker and Connell 1985). Since it has been shown that BCF is related to hydrophobicity (K_{ow}), then k_1 and k_2 may be individually related to K_{ow} (Hawker and Connell 1985).

If a contaminated organism is placed in a clean environment, the change in contaminant concentration within the organism is described by:

$$dC_a / dt = -k_2 C_a \qquad (7)$$

(Barron et al. 1990). When equation (7) is integrated:

$$C_a = C_a^{(t=0)} e^{-k_2 t} \quad (8)$$

or,

$$\ln C_a = \ln C_a^{(t=0)} - k_2 t \quad (9)$$

where $C_a^{(t=0)}$ is the amount of contaminant in the organism at the start of elimination. From equation (9), elimination rate can be calculated:

$$k_2 = (\ln C_a^{(t=0)} - \ln C_a) / t \quad (10)$$

Once k_2 is known, the time to steady state (TSS) can be determined:

$$t_{0.95} = -\ln 0.05 / k_2 \quad (11)$$

The biological half life (BHL) of the contaminant within the organism can also be calculated:

$$t_{0.5} = \ln 2 / k_2 \quad (12)$$

The elimination rate constant is a very important parameter for calibrating contaminant biomonitors. It can be used as a measure of persistence of a particular contaminant in an organism, which is essential for the establishment of that organism's tissue to environment contaminant concentration ratio (Clark et al. 1988).

A Quantitative Structure-Activity Relationship (QSAR) is a mathematical relationship between a chemical's behaviour (toxicity, activity) and its physical and chemical properties (Blum and Speece 1990). The elimination rates and bioconcentration factors of several hydrophobic organic chemicals have been shown to have an inverse linear relationship with log K_{ow} in a variety of aquatic organisms (Hawker and Connell 1986). In other words, the higher the log K_{ow} (hydrophobicity), the slower the elimination rate and the higher the BCF will be for a particular organism in which the above relationship holds. Non-polar, hydrophobic compounds preferentially partition

into the lipid phase, and have very limited solubilities in aqueous body fluids (blood, urine) that are the predominant routes of elimination. For this reason, these compounds will not be readily eliminated; the more hydrophobic the chemical, the slower its elimination will be from the organism (Lech and Vodicnik 1985).

Exposure to hydrophobic contaminants with slow elimination rates results in their bioaccumulation within an organism; if enough hydrophobic contaminants accumulate within an animal, non-specific adverse physiological effects occur due to the disruption of cell membrane and nerve functions (Veith et al.1983). These effects constitute narcosis, which is the reversible state of arrested activity of protoplasmic structures caused by a wide variety of organic chemicals (Veith et al. 1983). Some organisms have developed enzymatic processes that convert certain hydrophobic parent compounds into metabolites that are more water-soluble, facilitating their elimination (Lech and Vodicnik 1985). Such chemicals, once transformed, may not behave according to predictions or QSARs based on partition coefficients, such as K_{ow} (Spacie et al. 1983). This transformation also alters the compound's toxicological and pharmacological properties. A toxic parent compound may be detoxified, or turned into an equally toxic compound; a non-toxic parent compound may be transformed into an equally non-toxic metabolite, or activated to form a more toxic metabolite (Lech and Vodicnik 1985).

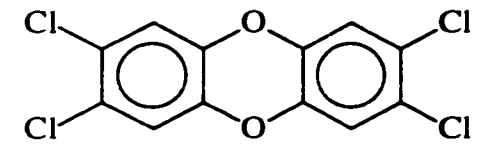
The Biological Fate of Organic Contaminants

The aryl hydrocarbon receptor pathway is an important component of the toxicological response of many living organisms to organic contaminants. Many of these contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAH) such as polychlorinated biphenyls (PCBs), are mediated through a protein known as the aryl hydrocarbon (Ah) receptor (Okey et al. 1994). The Ah receptor is widely distributed in various mammalian tissues, and also among non-mammalian vertebrates, but it may be absent from marine invertebrates (Hahn and Stegeman 1992). Studies on the phylogenetic distribution of the Ah receptor indicate an origin early in vertebrate evolution, before the divergence of bony and cartilaginous fish (Hahn and Stegeman 1992). This receptor has been detected in teleost and elasmobranch

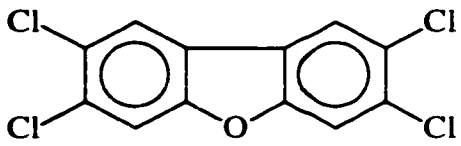
fish, but was not detectable in several agnathan fish (Hahn and Stegeman 1992). The Ah receptor has also been detected in amphibians (Marty et al. 1989).

The chemical having the greatest binding affinity to the Ah receptor is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Okey et al. 1994). Chemicals exhibiting high binding affinity to the Ah receptor have physical and chemical properties that are similar to TCDD. Such properties include molecular planarity, hydrophobicity, and similar steric, hydrogen-bonding and electrical properties (Okey et al. 1994). HAHs, such as chlorinated and brominated dibenzo-*p*-dioxins, dibenzofurans and non *ortho*-substituted biphenyls, exhibit physical and chemical similarities to TCDD, they all produce similar patterns of biochemical and toxic responses, and they all appear to exert their biological effects through binding to the Ah receptor (Figure 2) (Poland and Knutson 1982). The affinity with which a TCDD-like HAH binds to the Ah receptor has a major role in the determination of the evoked biochemical response (Okey et al. 1994). Chemical affinity to the Ah receptor depends on the properties of the ligand and the characteristics of the receptor of a particular host (Okey et al. 1994).

When a HAH enters a cell, it may bind to the Ah receptor, with the end result being the induction of the cytochrome P-450 monooxygenase or Mixed Function Oxidase (MFO) enzyme system (Poland et al. 1976). Many contaminants are able to induce *de novo* synthesis of cytochrome P450; the type of contaminant determines which cytochrome P450 enzyme is induced (Nerbert et al. 1989; Goksøyr and Förlin 1992). Chlorinated dibenzo-*p*-dioxins, certain planar PCBs, and PAHs are known to induce the 1A subfamily of cytochrome P450. One of the purposes of MFO activity is to decrease the hydrophobicity of non-polar organic chemicals in order to facilitate elimination from the organism (Neff 1979). MFO constitutes phase I activity, which involves the introduction of oxygen into the contaminant molecule through epoxidation, hydroxylation, dealkylation, and other oxidative, hydrolytic or reductive processes (Buhler and Williams 1989). Phase II activity, which acts on the products of phase I metabolism, involves conjugation with polar cellular constituents, such as glucuronic acid, sulfate, or glutathione (Buhler and Williams 1989). The Ah receptor has been shown to transcriptionally regulate cytochrome P450 1A1 and 1A2 (phase I enzymes),



2,3,7,8-tetrachlorodibenzo- *p*-dioxin



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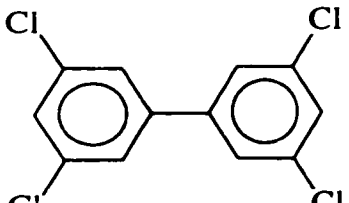

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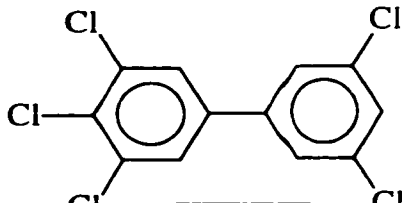

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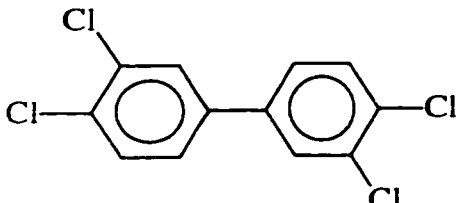

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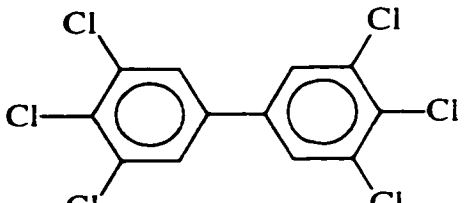


PCB 169

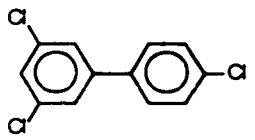
Figure 2. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and halogenated aromatic hydrocarbons with similar physical and chemical properties. The polychlorinated biphenyls (PCBs) illustrated achieve molecular planarity because they are unsubstituted in the *ortho* positions.

UDP-glucuronosyltransferase, glutathione S-transferase, NAD- and NADP-dependent quinone oxidoreductase, and aldehyde dehydrogenase (phase II enzymes) (reviewed in Nerbert et al. 1993). The Ah receptor pathway mediates all of the biological and toxicological effects of TCDD and related HAHs (Poland and Knutson 1982).

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls, or PCBs, are a group of chemicals characterized by a chlorinated biphenyl carbon ring configuration. The 209 possible PCB congeners differ in the number and substitution pattern of chlorine atoms on the biphenyl rings (Figure 3). PCBs are an environmental concern because of their prevalence, persistence, and hazard to humans and wildlife (Metcalfe and Haffner 1995). This group of chemicals is frequently found on priority chemical lists, such as the U.S. Environmental Protection Agency's Priority Pollutants list (Mackay 1991).

PCB residues have been detected in a wide variety of environments and organisms worldwide. A household / chemical waste dump in the Netherlands (Volgermeerpolder) is known to be contaminated with PCBs and other chlorinated contaminants (Belfroid et al. 1995). The sediments and waters of New Bedford Harbour (Massachusetts, USA) (Bergen et al. 1993) and some areas of coastal Georgia (USA) (Maruya and Lee 1998) have elevated levels of PCBs and other contaminants. PCBs have been detected in sediments and zebra mussels in the Lake St. Clair – Lake Erie corridor (Furlong et al. 1988; Morrison et al. 1995). Geyer et al. (1984) documented PCB contamination in the biota, waters, sediments and air of the Mediterranean basin. PCB residues have even been detected in deep sea biota at depths of greater than 1000 m (Stegeman et al. 1986). Lake Michigan sediments and sport fish are known to contain PCBs (Stow 1995). PCBs have been detected in channel catfish (*Ictalurus punctatus*), crappie (*Pomoxis spp.*), frogs (*Rana spp.*), heron (*Nyctanassa violacea*) and shad (*Dorosoma spp.*) collected from Louisiana watersheds (Dowd et al. 1985). Aroclor 1254, a commercial PCB mixture, persisted in pinfish (*Lagodon rhomboides*) and spot (*Leiostomus xanthurus*) for over 3 months (Hansen et al. 1971). PCBs and chlorinated pesticide residues have been detected in southern Ontario green frogs (*Rana clamitans*)



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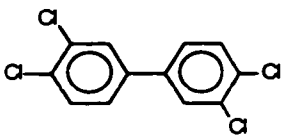

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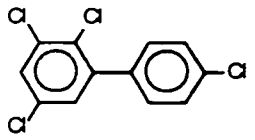

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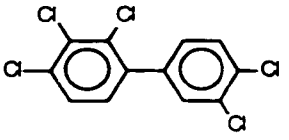

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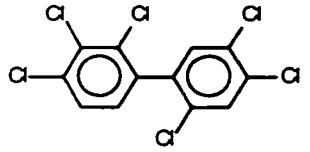

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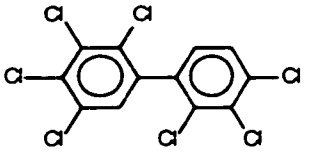

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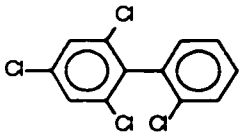

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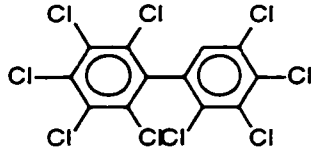

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Figure 3. Example of PCBs. Numbers represent PCB IUPAC designations. PCBs 39 and 77 are non *ortho*-substituted; PCBs 63 and 105 are mono *ortho*-substituted; PCBs 138 and 170 are di *ortho*-substituted; PCBs 50 and 206 are tri *ortho*-substituted.

and spring peepers (*Pseudacris cricufer*) (Russell et al. 1997a; Russell et al. 1995), as well as anurans collected from Yugoslavia (Vojinovic-Miloradov et al. 1996). PCB congeners have been detected in human breast milk (Larsen et al. 1994), adipose tissue (Mes et al. 1982) and blood (Fukano and Doguchi 1977).

Until the environmental hazards of PCBs were discovered, they were thought to be one of the most successful commercial chemical products. PCBs were patented in 1881 by Smith and Schulz, and their commercial production began in 1929; worldwide production has exceeded 2×10^9 kg (Hutzinger et al. 1974). PCBs were ideal as insulating coolants in electrical equipment because their highly non-polar nature results in a high dielectric constant (Hooper et al. 1990). They were also ideal as coolants, lubricants and preservatives because the stabilizing effects of chlorine's strong electronegativity on the biphenyl backbone resulted in heat resistance (Hooper et al. 1990). Commercial, industrial and domestic PCB use was widespread: they were used as electrical insulators, lubricants, hydraulic fuels, plasticizers and flame retardants, and as preserving agents in plastics, rubber, weatherproof coatings, varnishes, inks, and numerous other products (Hutzinger et al. 1974).

The properties that made PCBs commercially successful also rendered them serious environmental pollutants. The molecular stability of PCBs makes them resistant to physical and biological degradation. The ease with which PCBs are metabolized depends on certain molecular features (Niimi and Oliver 1983). In general, PCB degradation becomes more difficult with increasing chlorine substitution, especially when chlorine atoms occupy the *ortho* positions (Furukawa et al. 1978). The highly chlorine-substituted PCBs tend to be resistant to metabolism (Birnbaum 1985). Metabolism is facilitated if all the chlorine atoms are located on one of the biphenyl rings (Furukawa 1978). Also, the presence of two adjacent, non-chlorine substituted carbon atoms on one of the rings (termed vicinal hydrogen atoms) in the *meta-para* positions increases susceptibility to metabolism; *ortho-meta* vicinal hydrogens facilitate metabolism only if there is no more than one *ortho*-substituted chlorine atom on the molecule (Ballschmiter et al. 1978; Boon et al. 1987). Hutzinger et al. (1972) found that laboratory rats and Carneau pigeons were able to produce hydroxylated metabolites of a mono-, di- and tetrachlorinated biphenyl, but not of a hexachlorinated biphenyl congener. Safe et al.

(1976) detected metabolites of mono- and dichlorinated biphenyls administered by intraperitoneal injection in northern leopard frogs, and suggest that the metabolic processes responsible for chlorinated aromatic hydrocarbon metabolism in the frog are similar to that of mammalian systems.

The non-polar nature of PCBs conveys hydrophobicity, which is a very important factor in their environmental fate (Hooper et al. 1990). PCBs as a group have a wide range of hydrophobicities: their log K_{ow} values extend from 4.46 (monochlorobiphenyl) to 8.18 (decachlorobiphenyl) and are related to their molecular volumes (degree of chlorination) (Hawker and Connell 1988). Because of their hydrophobic nature, PCBs partition from polar (aqueous) environments into non-polar environments. Once inside an organism, PCBs will partition out of the aqueous phase (blood, excretory fluids) and into non-polar compartments, namely lipids. Because of their low solubility in aqueous excretory fluids, the elimination of PCBs from an organism will be extremely slow; PCBs will bioaccumulate unless the organism can metabolize them (Lech and Vodicnik 1985). PCBs present a human health hazard through chronic exposure, stemming from their potential to bioaccumulate and recalcitrance to biodegradation (Hooper et al. 1990).

The active biological and toxicological responses associated with PCBs are not the result of their direct activity. Some PCB congeners structurally resemble TCDD, a planar molecule which is one of the most potent oxidative enzyme inducers known (see Figure 2) (Newsted et al. 1995). PCBs that structurally resemble TCDD are able to achieve molecular planarity through unhindered rotation about the biphenyl bond. The presence of substituents in the *ortho* positions stearically hinders molecular planarity, hence PCB congeners that structurally resemble TCDD are non *ortho*-substituted (Poland and Knutson 1982).

Many of the toxic effects of these compounds are mediated through the Ah receptor. The resultant increase in MFO enzymes causes hydrophobic organic molecules (such as PAHs) to be transformed, resulting in intermediates and metabolites that could be potentially carcinogenic (see Figure 1) (McFarland and Clarke 1989; Foureman 1989; Landers and Bunce 1991; Nerbert et al. 1993). In a study conducted by Smeets et al. (1999), the exposure of PLHC-1 hepatoma cells (derived from *Poeciliopsis lucida*, a topminnow species) to benz(*a*)pyrene (a PAH) caused induction of cytochrome P-450

activity; pre-exposure of the cells to non *ortho*-substituted PCB 126 caused an 80-fold increase in cytochrome P-450 activity following benz(*a*)pyrene exposure. When northern leopard frogs (*Rana pipiens*) were injected with PCB 126, cytochrome P450-associated monooxygenase enzymes were induced, and their levels remained elevated for four weeks (Huang et al. 1998). Intraperitoneal injection of non-*ortho* PCBs 77 and 126 caused a dose-related induction of cytochrome P-450 activity in rainbow trout (*Oncorhynchus mykiss*) (Huuskonen et al. 1996). PCB 77 is also an estrogen mimic (Nesaretnam et al. 1996).

PCBs are an environmental health hazard of anthropogenic origin. Because of their persistence and bioaccumulation potential they are of ecological and toxicological concern.

Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (also known as polynuclear aromatic hydrocarbons, PAHs) are a group of chemicals consisting of two or more fused aromatic (benzene) rings (Figure 4). They are formed by natural synthetic processes (plant, bacterial and fungal biosynthesis), natural pyrolytic events (volcanoes and forest fires) and anthropogenic processes (Neff 1979). The burning of fossil fuels is an important source of PAH in the environment: the particulate fraction of vehicular exhaust contains significant quantities of PAHs; electrical generating facilities which burn fossil fuels produce liquid, solid and gaseous byproducts that are PAH-rich; solid residues (ash) from waste incineration contain high quantities of PAH (Neff 1979). PAHs are of environmental concern because of their prevalence and hazard to wildlife and human health. Like the PCBs, they are also commonly found on priority chemical lists, such as the U.S. Environmental Protection Agency's Priority Pollutants list (Mackay 1991). Generally, PAHs interact with cells in two ways to cause toxic responses: they may bind reversibly to lipid-rich sites and thereby interfere with cellular processes (narcosis), or their metabolites may bind covalently to cellular structures and macromolecules, causing long-term cellular damage (Neff 1979).

PAH congeners of toxicological concern are mobile, ranging in size from two to seven rings (Arfsten et al. 1996; EPA 1985). A large number of PAH congeners fall into

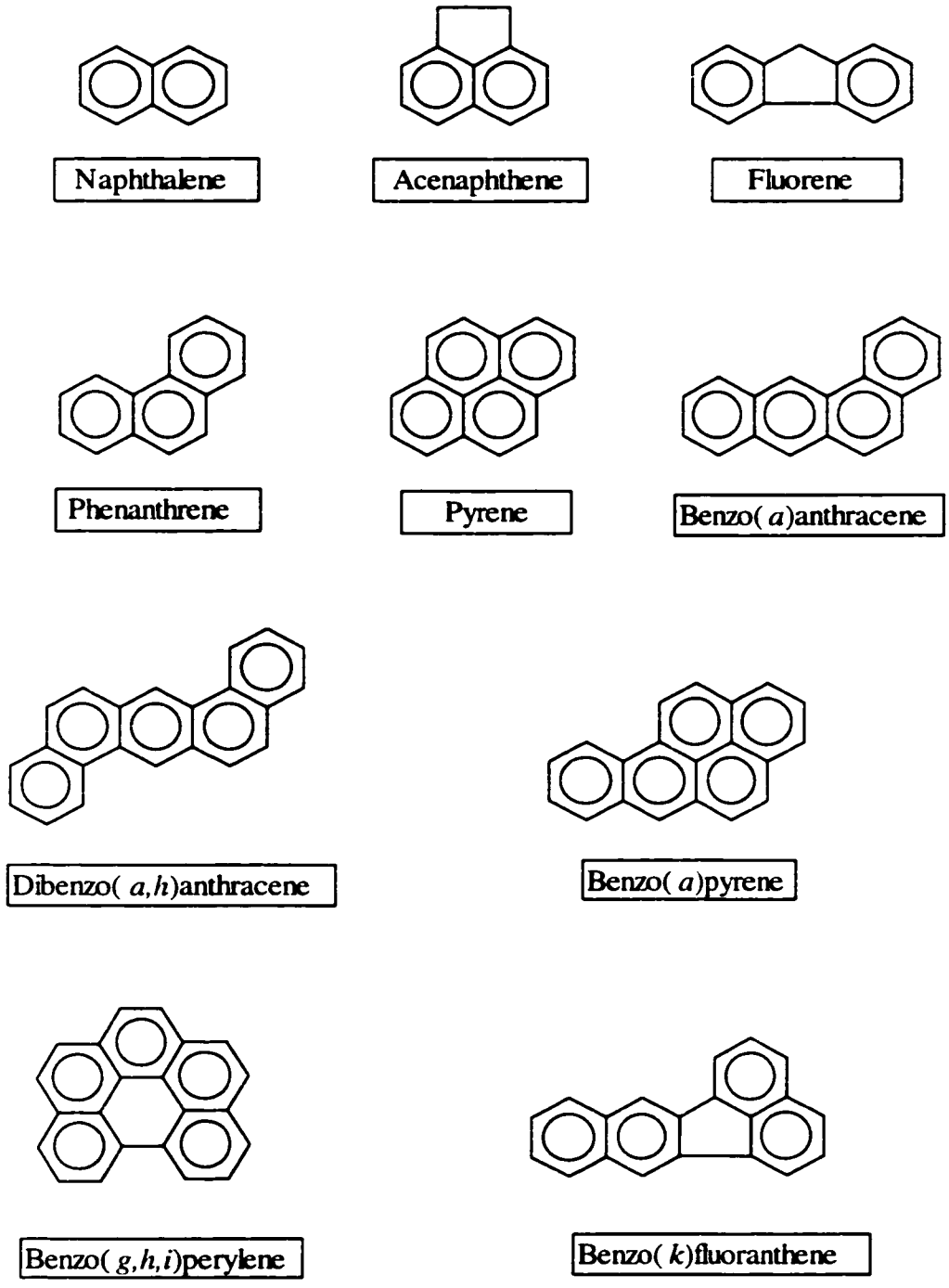


Figure 4. Example of polycyclic aromatic hydrocarbons (PAHs).

this range, varying in the number and position of aromatic rings, and in the number, chemistry and positioning of substituents on the rings (Neff 1979). PAHs have been detected globally in a wide variety of habitats and organisms, and have caused a variety of adverse effects. There are PAHs in the sediments of several Norwegian fjords (Naes et al. 1995). PAH residues have been detected in winter flounder, red hake, crabs and lobsters in New York, periwinkles, sponge and starfish in southern Norway, mussels in California, Scotland and British Columbia, clams and 14 species of fish in Italy, lobster from Nova Scotia, and diatoms from the Netherlands (reviewed in Meador et al. 1995). The sediments and mussels (*Mytilus galloprovincialis*) of the western Mediterranean Sea contained 1 to 20500ng/g and 25 to 390 ng/g PAHs, respectively (Baumard et al. 1998). The largest contribution to contaminant body burden in mussels and eels in the Rhine River comes from PAHs and PCBs (Hendriks et al. 1998). PAH residues have been detected in frogs from Yugoslavia (Vojinovic-Miloradov et al. 1996).

The hydrophobic nature of PAHs is an important factor in their environmental fate. Because of their low aqueous solubilities, dissolved or sorbed PAHs can be assimilated by organisms from the water or sediment (Eadie et al. 1982). Log K_{ow} values for PAHs range from 3.37 (naphthalene) to 6.75 (coronene) (Mackay et al. 1992).

PAHs are susceptible to environmental degradation through a variety of processes, most notably photo-oxidation (Arfsten 1996). Photo-oxidation involves the reaction of PAH with singlet oxygen, ozone, hydroxyl radical and other oxidizing agents, resulting in the formation of endoperoxides, diones and most importantly, quinones (Figure 5) (Neff 1979). These phototoxic products are formed within the organism, where they are subsequently reduced or oxidized (Arfsten 1996). This destroys the aromaticity of the affected benzene ring in the quinone, and yields an unpaired electron radical on the molecule. This radical can then interact with oxygen or peroxide to produce superoxide and hydroxyl radicals, which degrade proteins, deactivate enzymes and cause DNA damage (Neff 1979; Buhler and Williams 1989). PAHs exposed to ultraviolet light spectra commonly found in sunlight produce metabolites that have been shown to be toxic to a variety of organisms, including frogs (Ren et al. 1994; Ankley et al. 1994; Monson et al. 1995; Arfsten et al. 1996; Monson et al. 1999). PAHs vary in their sensitivity to photo-oxidation: resistance to photo-oxidation decreases with

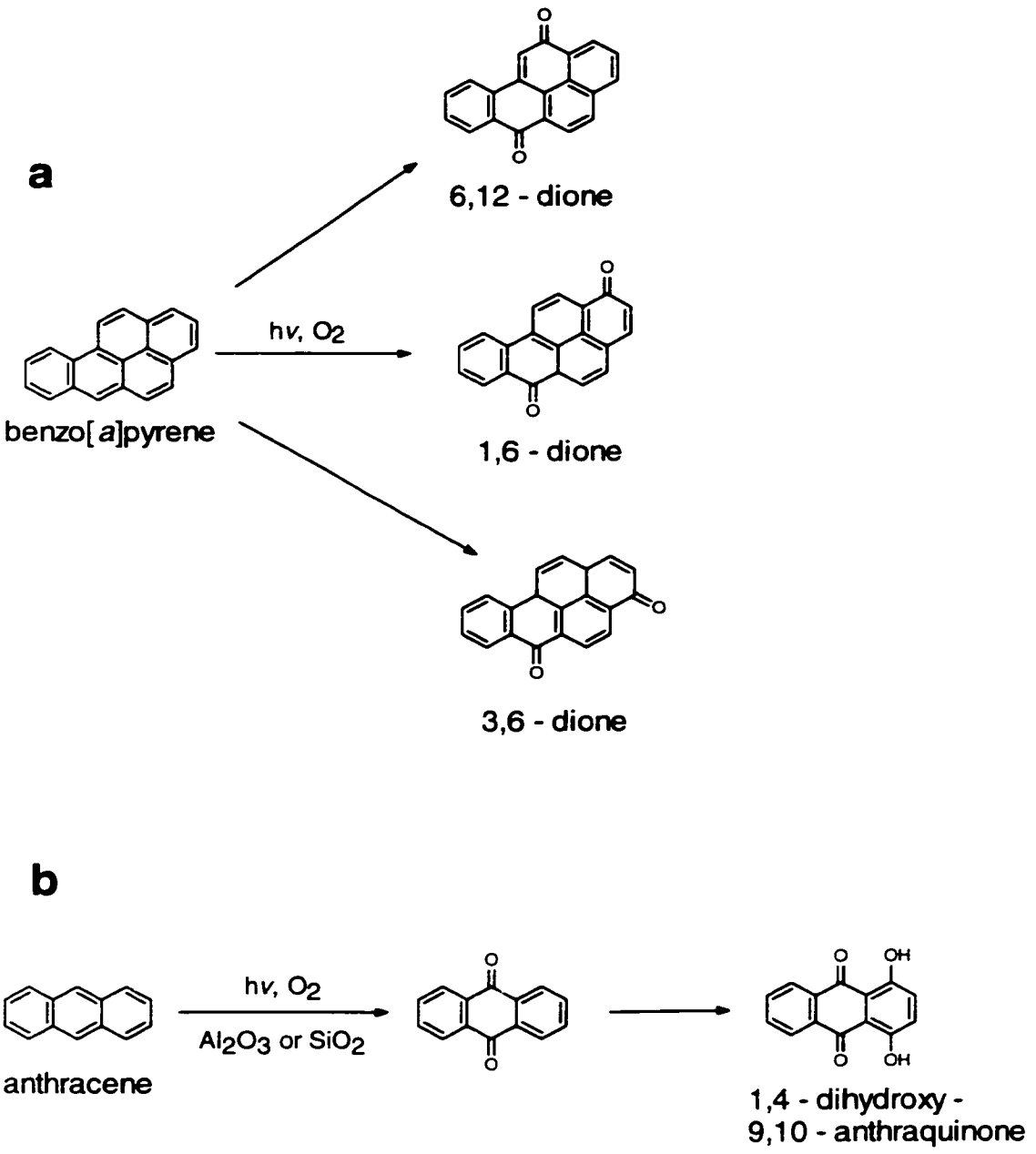


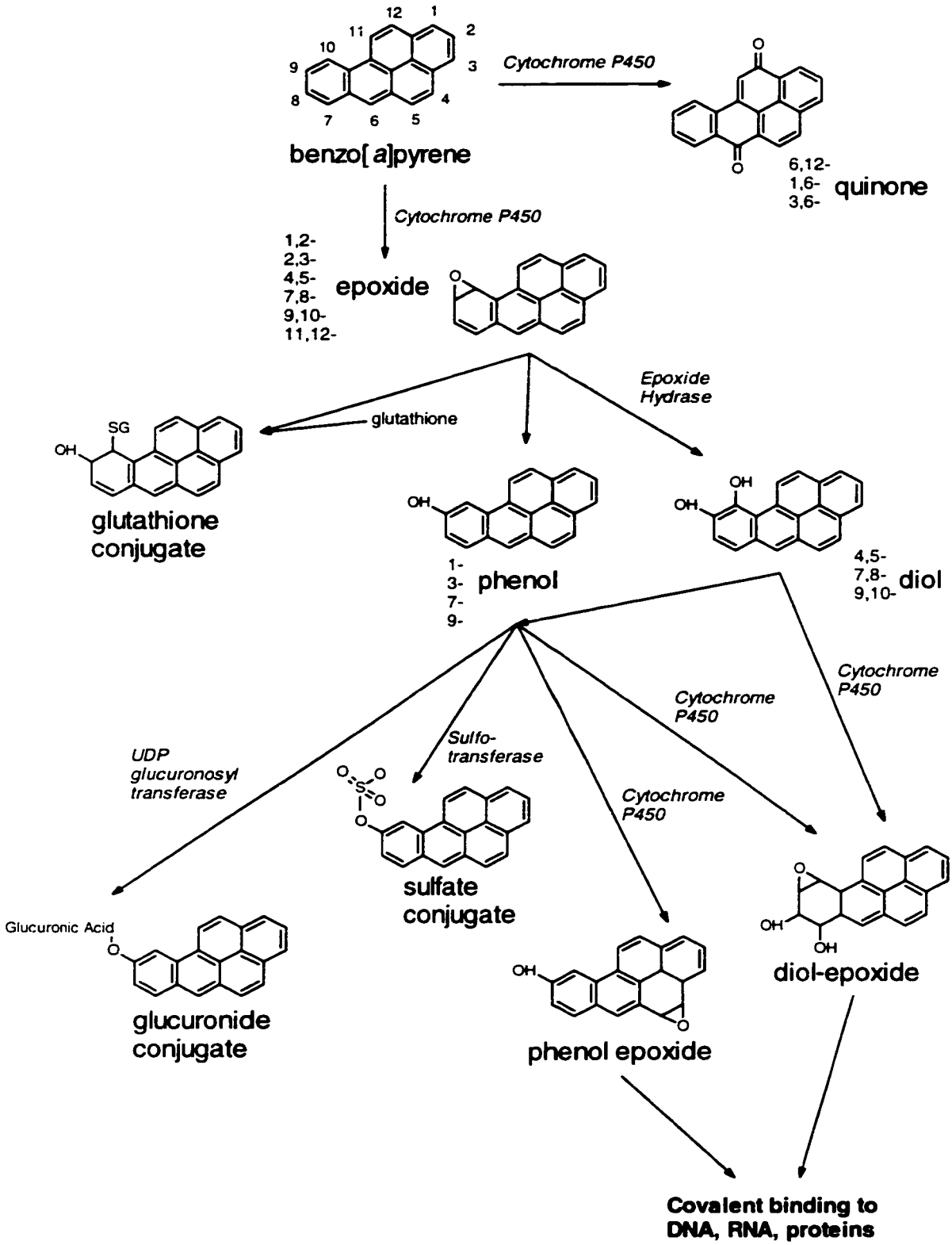
Figure 5. Photooxidation of benzo[*a*]pyrene to yield dione products (**a**). Photooxidation of anthracene adsorbed to silica gel or alumina to yield anthraquinone (**b**). Redrawn from Neff (1979).

increasing molecular weight (Neff 1979). In the aquatic environment, photo-oxidation rates decrease with increasing depth, due to the concomitant drop in light, temperature and oxygen concentration. Consequently, PAHs that come to rest in anoxic, dark or cold environments (such as lake sediments) are degraded slowly (Neff 1979). PAHs in sediments may be mobilized and made available to benthic invertebrates and fish (Clements et al. 1994).

PAHs can also be metabolically degraded by bacteria, protists, algae, fungi and animals (Cerniglia and Heitkamp 1989). The rates of PAH biodegradation are variable and dependent on PAH structure and the metabolic capability of the organism (Shuttleworth and Cerniglia 1995). In animals, the oxidative metabolism of organic, hydrophobic contaminants (such as PAHs) is primarily catalyzed by cytochrome P-450 dependent monooxygenases, which incorporate one atom of an oxygen molecule into the PAH (the other oxygen atom is reduced to water) (Cerniglia and Heitkamp 1989). An arene oxide intermediate is formed, which is further metabolized to yield diols, phenols, quinones and glutathione conjugates (phase I metabolism); these are further metabolized by phase II enzymes such as glutathione S-transferases, eopxide hydrase, sulfotransferases and UDP-glucuronosyltransferases (Cerniglia and Heitkamp 1989; Foureman 1989). In the metabolism of diols and phenols of PAHs, the cytochrome P450 system acts in concert with epoxide hydrase to produce diol epoxides, which can covalently bind to DNA, RNA and proteins; certain diol epoxides have been referred to as "ultimate carcinogenic species" (Figure 6) (Foureman 1989).

Indeed, several PAHs have been found to be carcinogenic, such as benz(*a*)pyrene, benz(*b*)fluoranthene and dibenz(*a,h*)anthracene (NRC 1983). Several studies found positive correlations between the occurrence of physiological abnormalities and the detection of PAHs and/or their metabolites; however, cause and effect has not been established. Leadley et al. (1998) found sediments highly contaminated with PAHs, PCBs and other organochlorine compounds in areas of the Detroit River, and that brown bullheads (*Ameiurus nebulosus*) collected from these areas exhibited high incidences of external abnormalities and hepatic lesions. Statistically significant correlations were found between PAH concentrations and the occurrence of hepatic lesions and neoplasms in various fish (reviewed in Baumann 1989). Metabolites of PAHs have been detected

Figure 6. Oxidative metabolism of benzo[*a*]pyrene. Initially, the molecule is oxidized by cytochrome P450-dependent oxidases to yield quinones and epoxides/arene oxides. The unstable arene oxides then undergo spontaneous rearrangement to form phenols, hydration via epoxide hydrolase to form diols, or conjugation with glutathione. The phenols and diols may then undergo conjugation with glucuronic acid (via UDP glucuronyl transferase) or sulfation (via sulfotransferase). The phenols and diols may also be further oxidized (via cytochrome P450) to yield multiple hydroxylated derivatives, most notably phenol epoxides and diol-epoxides, which may covalently bind to DNA, RNA and proteins. Redrawn from Varanasi et al. (1989b).


benzo[a]pyrene
Cytochrome P450
6,12-
1,6-
3,6- quinone
Cytochrome P450
1,2-
2,3-
4,5-
7,8-
9,10-
11,12- epoxide
glutathione
Epoxide Hydrase
SG
OH
glutathione conjugate
OH
1-
3-
7-
9- phenol
OH
OH
4,5-
7,8-
9,10- diol
UDP glucuronosyl transferase
Sulfo-transferase
Cytochrome P450
Cytochrome P450
Cytochrome P450
Glucuronic Acid
glucuronide conjugate
sulfate conjugate
OH
phenol epoxide
OH
OH
diol-epoxide
Covalent binding to DNA, RNA, proteins

in the bile of brown bullheads (*Ameiurus nebulosus*), and may account for the increased incidence of lesions (Maccubbin et al. 1988). Krahn et al. (1986) found a significant positive correlation between the occurrence of hepatic lesions and the concentration of PAH metabolites found in the bile of English sole (*Parophrys vetulus*). In addition, Varanasi et al. found that fish metabolize benz(*a*)pyrene to form intermediates that covalently bind to hepatic DNA and protein (Varanasi et al. 1989a). The clastogenic effect of benz(*a*)pyrene in the newt *Pleurodeles waltl* was shown to be related to the presence of benz(*a*)pyrene quinones in its tissues (Marty et al. 1989). Cancerous lesions were induced in newts (*Triturus cristatus*) by subcutaneous injection of several PAHs (Neukomn 1974). Subcutaneous implantation of benz(*a*)pyrene crystals in the tail of toad (*Bufo arenarum*) tadpoles caused papillomas, lymphomas and the development of an accessory tail or notochord (De Lustig and Matos 1971). Pacific herring eggs exposed to low concentrations of PAHs experienced malformations, genetic damage, decreased size and swimming ability, as well as mortality (Carls et al. 1999). The chemical teratogenesis of effluents from very weathered oil (from oil spills) to pink salmon (*Oncorhynchus gorbuscha*) embryos was associated with larger PAHs (Heintz et al. 1999). PAHs have also been shown to induce and elevate phase I metabolic activity, without increasing phase II metabolic activity, which could lead to the accumulation of highly carcinogenic and mutagenic products of phase I metabolism (Neff 1979). Salamanders from a PAH-contaminated lagoon in Texas displayed elevated phase I activity, while the 4,5-eopxide of benz(*a*)pyrene was not being metabolized by phase II enzymes, which may account for the high incidence of cancer in that population of salamanders (Rose 1977).

PAHs are plentiful in today's environment. Because of their low aqueous solubilities, PAHs can accumulate in aquatic food webs (Landrum 1982; Neff 1985). Their degradation form intermediates and metabolites that are potentially carcinogenic (Foureman 1989; Nerbert et al. 1993). Because of their ubiquity and potential health hazards, PAHs are of ecological and toxicological concern.

Methodology and Experimental Design

Animal Maintenance

Northern leopard frogs (*Rana pipiens*) were ordered from the Wm. Lemberger Co. (Wisconsin, USA). While in captivity and over the duration of the experiment, they were fed approximately 3 live crickets every two days (obtained from Pro-Fish Centre, Windsor, Ontario).

The frogs were kept in six 40-gallon aquaria that were each divided into three cells by two permeable partitions. The aquaria were filled approximately 5 cm deep with dechlorinated water, and beach pebble was provided as a terrestrial substrate. Submersible charcoal filters (one per aquarium) were used to gently circulate the water, and to adsorb organic contaminants. Additional adsorption capacity was provided by small nylon bags filled with charcoal (1 per cell). Aquarium water was changed weekly. Over the duration of the study, aquarium water temperature remained constant at approximately 19.5°C.

Chemical Delivery

The PCBs used in this experiment consisted of a 1:1:1 mixture of Aroclors 1242, 1254 and 1260, obtained from the Canadian Wildlife Service Research Centre (Hull, Quebec, Canada). The PAH mixture used in this experiment contained many of the PAH congeners listed on the U.S. Environmental Protection Agency's Potential Contaminants of Concern (EPA 1985). This mixture was obtained form Accustandard (Connecticut, USA), and consisted of the following 16 congeners, present in equal concentrations:

naphthalene	benz(*a*)anthracene
acenaphthylene	chrysene / triphenylene
acenaphthene	benz(*b*)fluoranthene
fluorene	benz(*k*)fluoranthene
phenanthrene	benz(*a*)pyrene
anthracene	indeno(*1,2,3-c,d*)pyrene
fluoranthene	dibenz(*a,h*)anthracene
pyrene	benz(*g,h,i*)perylene

The PCB and PAH mixtures used in this experiment were transferred out of their storage solvent (2,2,4-trimethylpentane) and into sunflower oil for injection. Mixtures of PCB or PAH congeners were used to better emulate chemical mixtures to which organisms are

exposed in the environment, and to cover a range of physicochemical properties such as octanol-water partition coefficients, chlorine substitution and number of aromatic rings.

The chemicals were introduced into the frogs by intraperitoneal injection (Coristine et al. 1996). Intraperitoneal injection was used to avoid differential congener absorption that would result when using dietary means of introducing the chemicals into the frogs (Russell et al. 1995b). The masses of the frogs were recorded immediately prior to injection. A group of 18 frogs were injected with PCB-spiked sunflower oil to a total body concentration of 1μg (total PCBs) / g frog (treatment 1). A second group of 18 frogs were injected with PAH-spiked sunflower oil to a total body concentration of 2μg (each PAH congener) / g frog (treatment 2). A third group of 18 frogs was injected with an unspiked sunflower oil blank (treatment 3 – control). All injection volumes were constant per unit frog weight. The contaminant concentrations injected were similar to those found in sediments and brown bullheads (*Ameiurus mebulosus*) of the Detroit River (Leadley et al. 1998), and chosen on the basis of environmental relevance and to accommodate analytical detection limits.

Post-Injection and Sampling Regime

Immediately following injection, the long fourth toe on one of the hind legs was clipped for identification: PAH-injected frogs had their right toes clipped and PCB-injected frogs had their left toes clipped. Toes were not clipped on blank-injected (control) frogs. During the course of the study, each aquarium cell held three frogs, one from each treatment group. In this manner, each individual frog could be monitored over the duration of the study (Figure 7).

The 6 aquaria, each subdivided into 3 cells with 2 permeable partitions, formed a 6x3 matrix. Six sampling times were then randomly assigned to the cells of the aquaria such that none of the aquaria received more than one replicate sample time. Thus, interspersion was forced across the cells of the six aquaria (Hurlbert 1984). The overall experimental design consisted of a *split plot*, with the six sampling times comprising the "whole plot" treatments and the three intraperitoneal injections the "split-plot' treatments.

At each sampling time, nine frogs (three replicates from each treatment group) were collected from three cells. Frogs were weighed and sacrificed by succumbing to

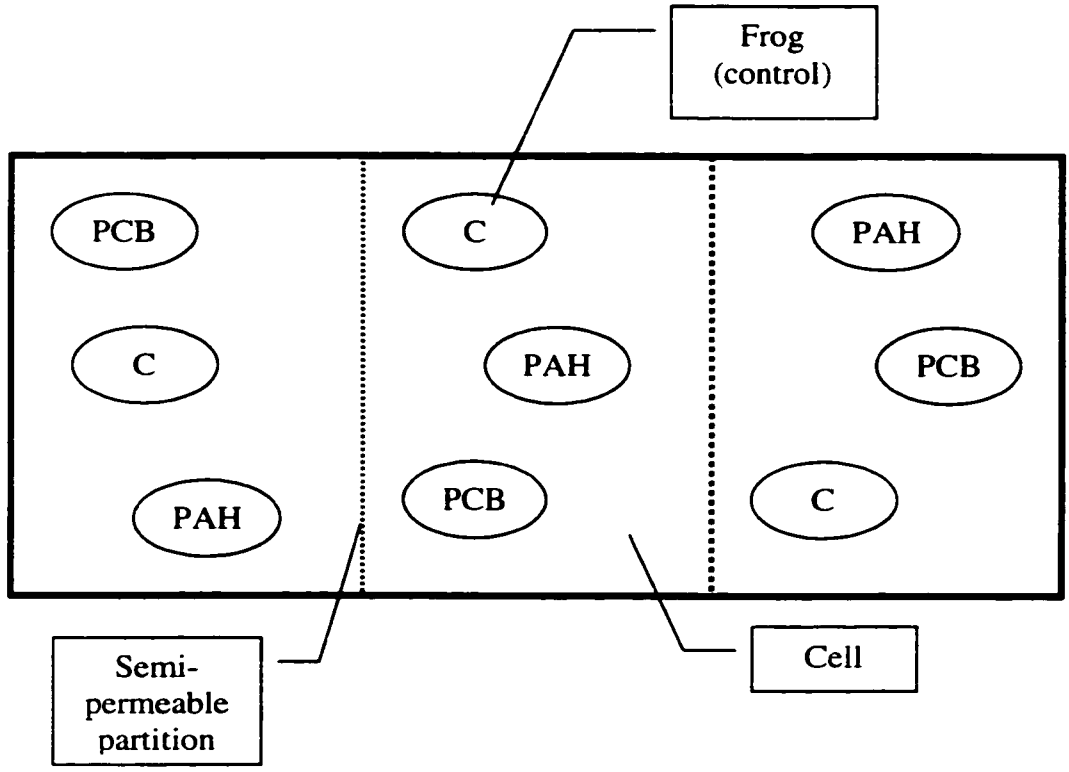


Figure 7. Schematic drawing of one aquarium, divided into three cells by two semi-permeable partitions. Each cell contained three frogs: one from the PAH injection group (PAH), one from the PCB injection group (PCB) and one from the blank (control) injection group (C). A total of six such aquaria were used in this study.

cold conditions (ice water) 24 hours, 48 hours, 4 days, 8 days, 16 days and 24 days following injection (Canadian Council on Animal Care 1969). They were kept frozen (-20°C) in hexane-rinsed aluminum foil until later analysis. Refer to Figure 8 for an overview of the harvesting regime and subsequent chemical analysis.

Chemical Extraction and Clean-Up

Prior to extraction, the frogs were thawed and homogenized whole. All glassware and laboratory utensils were soaked and washed in warm soapy water, rinsed with hot tap water, and solvent-rinsed with acetone and hexane. The extraction of organic contaminants from biological tissue followed the protocol presented in Lazar et al. (1992):

A 5g subsample of the homogenized frog was further homogenized with granular anhydrous sodium sulphate (ACS grade, VWR Scientific, Mississauga, Ontario, Canada) using a glass mortar and pestle. Once the moisture from the sample was absorbed, the mixture was poured into a 35cm x 2cm glass column that was previously plugged with glass wool and filled with 1:1 dichloromethane:hexane (Omnisolv grade, VWR Scientific) and anhydrous sodium sulphate. The sample was spiked with a reference standard (1mL of 25 µg/kg chlorinated benzenes) (Accustandard, Conneticut, U.S.A.) to determine extraction efficiency. After soaking for one hour, the sample was eluted with additional dichloromethane:hexane. The resulting elute was roto-evaporated to approximately 5mL, and made up to 25mL with hexane.

For lipid determination, 2mL of the above extract was pipetted into a pre-weighed beaker, evaporated to dryness, and placed into a drying oven for one hour at 105°C. After cooling in a dessicator, the beaker was re-weighed. The difference in weight represents the weight of lipid in the sample. Percent of lipid in the sample was calculated as follows:

$$\% \text{ lipid} = (M_L / M_T \times V_T / V_e) \times 100$$

where M_L = Mass of lipid
M_T = Mass of sample
V_T = Total volume of extract (25mL)
V_e = Volume of extract used for lipid determination (2mL)

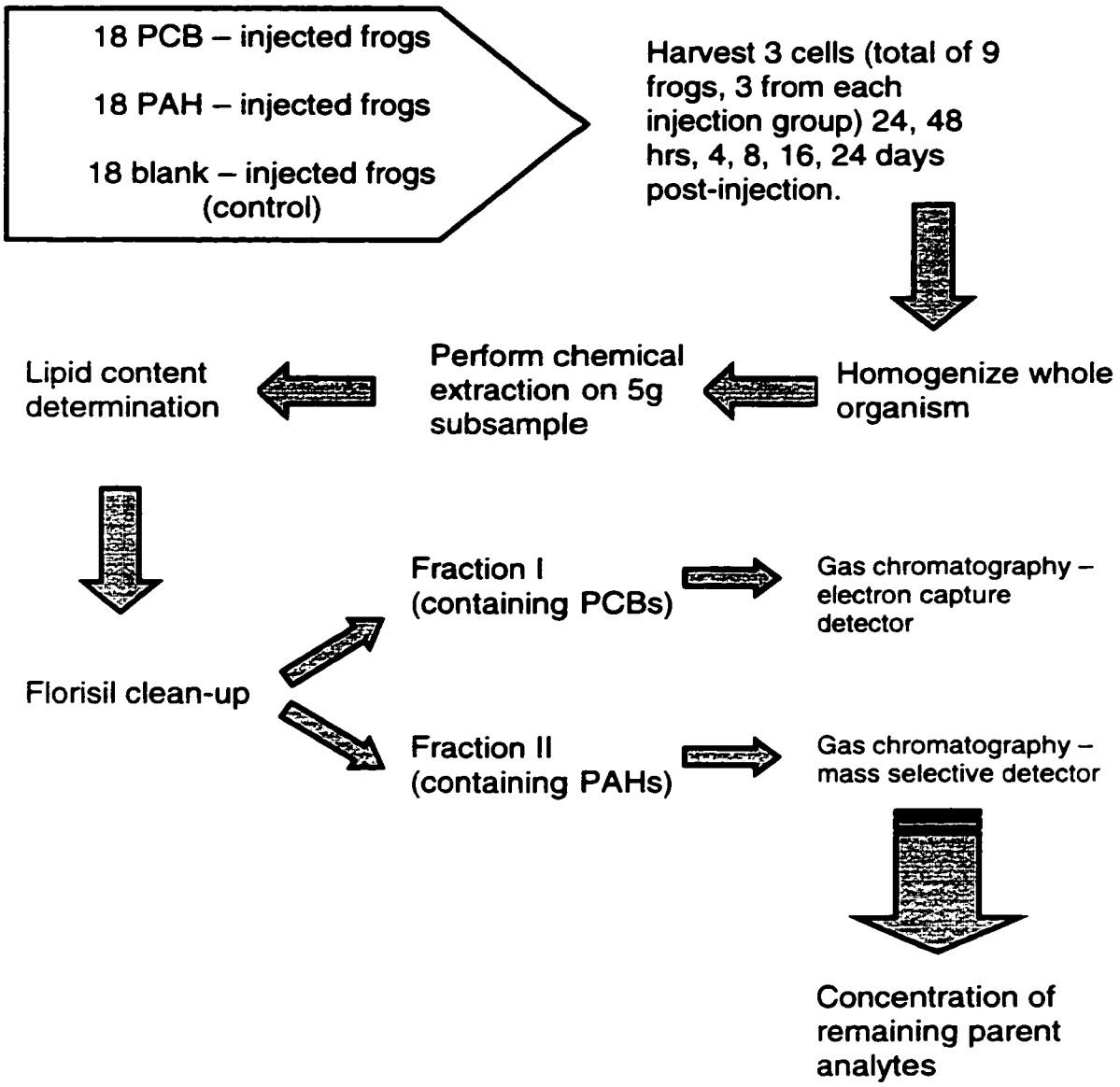


Figure 8. Schematic drawing depicting harvesting regime and chemical analysis. Sacrificed frogs were homogenized whole. A 5g subsample was then used for chemical extraction with 1:1 dichloromethane : hexane. The extract was analysed for lipid content, and eluted through Florisil (clean-up). The first fraction (eluted with hexane) was analysed by gas chromatography – electron capture detector, which gave the concentration of PCBs in the frog tissue. The second fraction (eluted with 60:40 dichloromethane : hexane) was analysed by gas chromatography – mass selective detector, which gave the concentration of PAHs in the frog tissue.

The remaining 23mL of sample extract was roto-evaporated to approximately 2mL, and pipetted into a 25cm x 1cm glass column for florisil clean-up. In instances where lipid content was high (greater than 15%), the remaining 23mL of sample extract (after determination of lipid content) underwent lipid separation by gel permeation chromatography (GPC) as follows:

The remaining 23mL of sample extract was roto-evaporated to 1.5-2mL. 2mL of dichloromethane were added, and the resultant solution transferred to the top of a GPC column, previously packed with glass wool and a slurry of BioBeads (50g) (SX-3, BioRad) in 1:1 dichloromethane:hexane. The sample was allowed to sink into the BioBeads, and elution was initiated. The initial 150mL of elute was discarded, and the final 150mL elute was retained, roto-evaporated to 2mL, and transferred to a florisil column for clean-up.

For clean-up, the column was previously plugged with glass wool, filled with hexane and 6g of activated Florisil® (60-100 mesh, VWR Scientific), and topped with 2cm of anhydrous sodium sulphate. The first fraction (containing PCBs) was eluted using 50mL hexane. When the hexane reached the top of the sodium sulphate, the flask containing the first fraction was replaced with another flask, and the second fraction (containing PAHs) was then eluted with 60:40 dichloromethane:hexane. 5mL of 2,2,4-trimethylpentane (Omnisolv grade, VWR Scientific) was added to the collected fractions, they were roto-evaporated to approximately 2mL, and then they were transferred to volumetric flasks and made up to an appropriate volume with 2,2,4-trimethylpentane.

Chemical Analyses

Fraction I (containing PCBs) was analysed using gas chromatography with an electron capture detector (ECD). A Hewlett Packard model 5890 - Series II Plus gas chromatograph, equipped with an integrator (HP-3396), autosampler (HP-7673A), and an electronic pressure controller was used. A ^{63}Ni electron capture detector was used to analyse Fraction I (containing PCBs) for this study. The chromatography column was 60m x 0.25mm (internal diameter) x 0.25μm DB-5 film. The injector temperature was 250°C, and the detector temperature was 320°C. The carrier gas was ultra-high purity helium, flowing at 1mL/min at 23.5psi. The make-up gas was 95:5 argon:methane

flowing at 50mL/min. 1μL sample was injected in splitless injection mode. The initial oven temperature was 90°C for 30 seconds. The temperature increased at a rate of 10°C/min to 200°C, and then the rate of temperature increase was reduced to 2.5°C/min until 275°C was reached. The final temperature was held for 2 minutes. The analytical detection limit for this procedure was 0.05 ng g^{-1}.

Fraction II (containing PAHs) was analysed using gas chromatography with a mass selective detector (MSD). A Hewlett Packard model 5890 / 5970 GC - MSD, equipped with an HP7673A autosampler was used to analyse Fraction II for this study. The chromatography column was 30m x 0.25mm (internal diameter) x 0.25μm DB-5 film. The injector temperature was 250°C, and the GC - MSD interface temperature was 280°C. The carrier gas was ultra-high purity helium, flowing at 1mL/min, with a column head pressure of 13psi. 1μL sample was injected in splitless injection mode. The initial oven temperature was 80°C for 1 minute. The temperature increased at rate of 20°C/min to 200°C, and then the rate of temperature increase was reduced to 5°C/min until 280°C was reached. The final temperature was held for 10 minutes. The detection mode used for the MSD was electronic impact, selected ion monitoring, with a dwell time of 50 – 75 msec per ion. The analytical detection limit for this procedure was 100 ng g^{-1}.

The concentration of individual congeners was determined using the following equation:

$$C_{sam} = A_{sam} / A_{std} \times StdC_{org} \times V / M$$

where: C_{sam} = the concentration of a particular congener in the sample
A_{sam} = the peak area of the congener in the sample
A_{std} = the peak area of the congener in the standard
$StdC_{org}$ = the concentration of the congener in the standard
V = final volume of the sample (mL)
M = mass of sample (g)

Statistical Analyses

A multivariate analysis of variance (MANOVA) was used to test for inconsistencies in initial and final frog weights and frog lipid contents among the injection treatment groups (MANOVA#1). The independent variables were time,

injection treatment, and treatment x time interaction. Initial weight, final weight and lipid content were the dependent variables. *A priori* contrasts between injection treatments compared PCB and PAH-treated frogs, and each of these to the control. In addition, the control-injected treatment was tested for concentration changes over time.

The PCB and PAH congeners used in subsequent analyses, as well as their molecular structures and hydrophobicities (log K_{ow}) are presented in Tables 1 and 2, respectively. Data were not corrected for extraction efficiency, and values below analytical detection limits were excluded from linear regression analyses. Wet weight concentrations of individual congeners in the frogs were lipid-normalized. To correct for possible contaminant cycling, data were control- corrected by subtracting lipid-normalized control concentrations from treated concentrations by replicate for each sampling time, followed by ln-transformation.

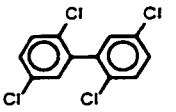
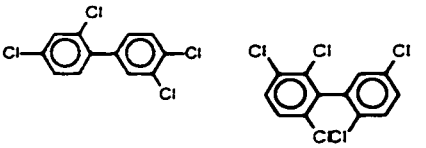
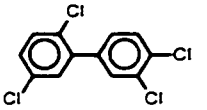
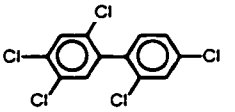
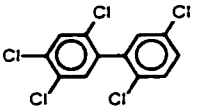
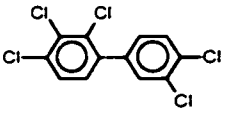
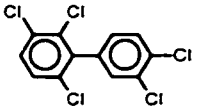
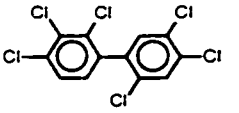
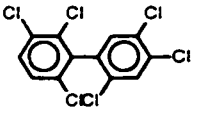
Since observations on each frog consisted of 16 PCBs and 16 PAHs, a MANOVA was used to account for the underlying experimental design and test for:

a) Inconsistencies in the concentration-time relationship between the control-corrected PCB and PAH injection treatments;
b) Overall trends (linear or curvilinear)in concentration over time (averaged over injection treatments);
c) Overall differences between injection treatments (averaged over the sampling times).

(MANOVA#2). The independent variables were time, injection treatment, and treatment x time interaction. The dependent variables were the congener concentrations. To have sufficient degrees of freedom for this MANOVA, congeners were combined into 8 groups (4 PAH groups, 4 PCB groups) with 4 PCB or PAH congeners per group. Congeners were grouped according to log K_{ow}, such that the 4 PAH congeners with the lowest log K_{ow}s were grouped together, and so forth.

Elimination rate constants were estimated from the slopes of linear regressions of lipid-normalized, control-corrected, ln-transformed congener concentration vs. time. Times to steady state (TSS) and biological half-lives (BHL) were determined, where appropriate, using the elimination rates estimated above with equations (11) and (12) in the Background chapter, respectively. Individual linear regressions of lipid-normalized,

Table 1. Chemical and IUPAC names of the PCBs analysed in this study, their structures, and log K_{ow} values (from Hawker and Connell 1988).

PCB congener	Structure	Log K_{ow}
2,2',5,5'-tetrachlorobiphenyl (PCB 52)		5.84
2,3',4,4'-tetrachlorobiphenyl / 2,2',3,5,6' pentachlorobiphenyl (PCB 66 / 90)		6.20
2,3',4',5-tetrachlorobiphenyl (PCB 70)		6.20
2,2',4,4'5-pentachlorobiphenyl (PCB 99)		6.39
2,2',4,5,5'-pentachlorobiphenyl (PCB 101)		6.38
2,3,3',4,4'-pentachlorobiphenyl (PCB 105)		6.65
2,3,3',4',6-pentachlorobiphenyl (PCB 110)		6.48
2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138)		6.83
2,2',3,4',5',6-hexachlorobiphenyl (PCB 149)		6.67

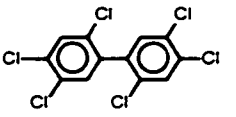
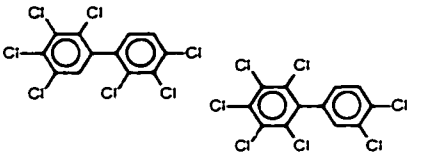
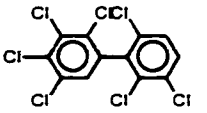
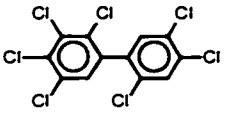
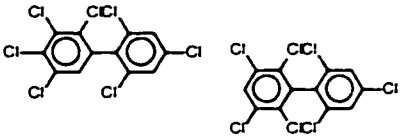
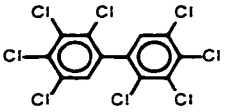
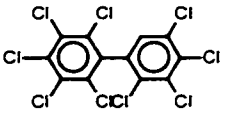
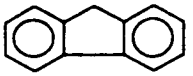
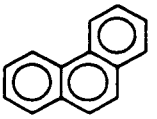
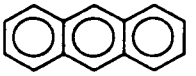
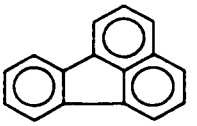
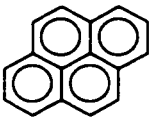
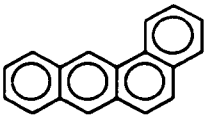
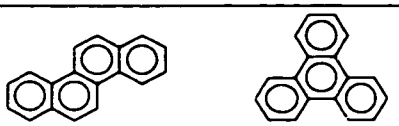
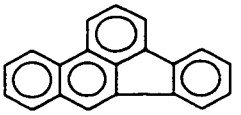
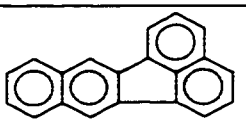
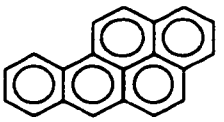
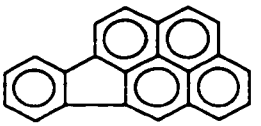
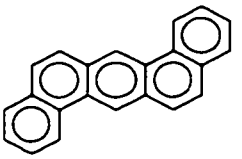
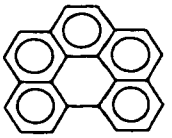
PCB congener	Structure	Log K_{ow}
2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)		6.92
2,2',3,3',4,4',5-heptachlorobiphenyl / 2,3,3',4,4',5,6-heptachlorobiphenyl (PCB 170 / 190)		7.46
2,2',3,3',4,5,6'-heptachlorobiphenyl (PCB 174)		7.11
2.2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180)		7.36
2,2',3,4,4',5,6'-heptachlorobiphenyl / 2,2',3,4',5,5',6-heptachlorobiphenyl (PCB 182 / 187)		7.20
2,2',3,3',4,5,5',6-octachlorobiphenyl (PCB 194)		7.80
2,2',3,3',4,5,5',6,6'-nonachlorobiphenyl (PCB 206)		8.09

Table 2. Chemical names and abbreviations of the PAHs analysed in this study, their structures, and log K_{ow} values (from Mackay et al. 1992 and Maruya et al. 1996).

PAH congener	Structure	Log K_{ow}
Naphthalene (NA)		3.37
Acenaphthylene (AL)		4.00
Acenaphthene (AE)		3.92
Fluorene (FL)		4.18
Phenanthrene (PHE)		4.57
Anthracene (AN)		4.54
Fluoranthene (FLT)		5.22
Pyrene (PY)		5.18
Benzo(*a*)anthracene (BAA)		5.91

PAH congener	**Structure**	**Log K_{ow}**
Chrysene / Triphenylene (C/T)		5.49
Benzo(*b*)fluoranthene (BBF)		5.80
Benzo(*k*)fluoranthene (BKF)		6.00
Benzo(*a*)pyrene (BAP)		6.04
Indeno(*1,2,3-c,d*)pyrene (IP)		6.50
Dibenzo(*a,h*)anathracene (DAA)		6.75
Benzo(*g,h,i*)perylene (BPY)		6.50

control-corrected, ln-transformed congener concentrations on time were performed using the General Linear Model (GLM) procedure of the SAS statistical package. Estimates of slope in the linear regressions were negative-transformed so that elimination rates would be expressed as a positive value.

To determine if the elimination rates of a) PCB or b) PAH congeners were influenced by their hydrophobicities (log K_{ow}) in northern leopard frogs, linear regressions of PCB and PAH elimination rates on log K_{ow} were performed. PCB and PAH elimination rates determined in this study were compared with those determined in other studies using other organisms.

To determine if there was a difference between the elimination rate-log K_{ow} relationships for PCBs and PAHs in northern leopard frogs and to deduce which chemicals were being metabolized, the PCB / PAH elimination rate – log K_{ow} relationships were compared. Differences between individual congener elimination rates in relation to log K_{ow} were investigated and compared with data from other studies.

Results

Experimental Design MANOVAs

Throughout the study, frogs manifested no skin lesions, anorexia, or mortality. Frogs were fed for the duration of the study. Results of MANOVA#1 indicated that there were no inconsistencies with initial and final frog weights and lipid content (Table 3). The results of specific tests used within this MANOVA are as follows:

- Averaging across injection treatments, initial frog weights, final weights and lipid content did not show any significant (probability, $p<0.05$) changes over time ($p=0.495$, 0.29, 0.18 respectively).
- Averaging across sample times, no significant differences in initial weights, final weights and lipid content were detected among the injection treatments ($p=0.61$, 0.98, 0.4 respectively).
- No significant differences in initial and final weights and lipid content were detected when comparing PCB and PAH-injected groups ($p=0.62$, 0.88, 0.85 respectively). Comparison of PCB and control-injected groups yielded similar results ($p=0.62$, 0.97, 0.22 respectively). Likewise, comparison of PAH and control-injected groups yielded no significant differences ($p=0.32$, 0.86, 0.29 respectively).
- None of the injection treatments showed a significant interaction between initial weight and time ($p=0.52$), final weight and time ($p=0.5$) and lipid content and time ($p=0.18$).

Average analyte recovery (a measure of extraction efficiency) was 97.6% (± 1.2% standard error). Analyte concentrations in food and control frogs are summarized in Table 4. The variability of congener concentrations among replicates of each sampling time reflects human error associated with injection and laboratory analytical precision, and the individual variation in the frogs' abilities to eliminate the analytes. The presence of the occasional detectable amount of naphthalene in control and PAH-injected frogs could be the result of external contamination during sample processing. MANOVA revealed a marginally significant effect of time on analyte concentrations in the control group ($p=0.03$), which may be due to analyte cycling or the possible presence of baseline amounts of these analytes in the frogs prior to the initiation of the study (Table 5). The use of control-corrected data in subsequent analyses should abate against these effects.

Table 3. Results of MANOVA testing for inconsistencies in initial and final frog mass and lipid content.

Test	Wilks' Lambda	F Value	Numerator Degrees of Freedom	Denominator Degrees of Freedom	Probability
Overall effect of **time** on:					
• Mass (initial)	0.721	0.93	5	12	0.495
• Mass (final)	0.631	1.40	5	12	0.29
• Lipid Content	0.564	1.86	5	12	0.18
Overall effect of **injection treatment** on:					
• Mass (initial)	0.959	0.51	2	24	0.61
• Mass (final)	0.998	0.02	2	24	0.98
• Lipid Content	0.927	0.94	2	24	0.40
Contrasting PCB and PAH **injection treatments**:					
• Mass (initial)	0.989	0.26	1	24	0.62
• Mass (final)	0.999	0.02	1	24	0.88
• Lipid Content	0.999	0.03	1	24	0.85
Contrasting PCB and Control **injection treatments**:					
• Mass (initial)	0.99	0.25	1	24	0.62
• Mass (final)	1.00	0.001	1	24	0.97
• Lipid Content	0.937	1.61	1	24	0.22
Contrasting PAH and Control **injection treatments**:					
• Mass (initial)	0.959	1.02	1	24	0.32
• Mass (final)	0.999	0.03	1	24	0.86
• Lipid Content	0.953	1.18	1	24	0.29
Injection treatment x time **interaction**:					
• Mass (initial)	0.721	0.93	10	24	0.52
• Mass (final)	0.713	0.97	10	24	0.5
• Lipid Content	0.608	1.55	10	24	0.18

Table 4. Summary of lipid-normalized analyte concentrations in food, control frogs, and treated frogs harvested 24 hours after injection. **A**: PCBs; **B**: PAHs.

A

Analyte	Log K_{ow}	Average Concentration in Food (ng/g)	Highest Concentration in Blank (ng/g)	Average Concentration 24 hours post PCB treatment (ng/g)
PCB 52	5.84	0	0	678.01
PCB 66/95	6.17	5.76	73.08	742.4
PCB 70	6.2	0	32.99	385.57
PCB 99	6.39	0	15.65	269.44
PCB 101	6.38	0	44.2	803.84
PCB 105	6.65	0	4.01	320.66
PCB 110	6.48	0	6.28	562.44
PCB 138	6.83	5.22	11.67	1015.37
PCB 149	6.67	0	2.47	782.65
PCB 153	6.92	8.28	15.8	771.35
PCB 170/190	7.37	0	2.86	374.93
PCB 174	7.11	0	0	347.55
PCB 180	7.36	3.57	6.36	750.79
PCB 182/187	7.19	1.43	3.7	413.98
PCB 194	7.8	0	0.81	145.59
PCB 206	8.09	49.08	0	43.35

B

Analyte	Log K_{ow}	Average Concentration in Food (ng/g)	Highest Concentration in Blank (ng/g)	Average Concentration 24 hours post PAH treatment (ng/g)
Naphthalene	3.37	19.03	5816.07	0
Acenaphthylene	4	0	0	3301.3
Acenaphthene	3.92	0	0	31395.41
Fluorene	4.18	1.57	1152.67	11951.74
Phenanthrene	4.57	10.47	1617.15	12556.35
Anthracene	4.54	0	0	4444.67
Fluoranthene	5.22	7.02	1563.43	15149.51
Pyrene	5.18	3.69	0	12840.06
Benz(*a*)anthracene	5.91	0	0	13881.69
Chrysene / Triphenylene	5.49	1.18	0	17193.46
Benz(*b*)fluoranthene	5.8	0	1393.42	29486.58
Benz(*k*)fluoranthene	6	0	0	8918.8
Benz(*a*)pyrene	6.04	4.86	0	0
Indeno(1,2,3-*c,d*) pyrene	6.5	0	0	9719.87
Dibenz(*a,h*)anthracene	6.75	0	0	10080.41
Benz(*g,h,i*)perylene	6.5	39.97	0	5845.06

Table 5. Results of MANOVA testing for changes in analyte concentration over time in the Control injection group.

Wilks' Lambda	F Value	Numerator Degrees of Freedom	Denominator Degrees of Freedom	Probability
0.005	2.02	35	27.67	0.03

The results and related statistics of MANOVA#2 are summarized in Table 6. Averaging across the injection treatments, control-corrected, ln-transformed chemical concentrations declined through the 6 sampling times in a linear fashion ($p=0.038$). As might have been expected, the relative concentrations differed between control-corrected PCB and PAH injection treatments, averaging over the sampling times ($p=0.0001$). Concentration trends over time (the slope of the relationship) were different between the PCB and PAH treatments ($p=0.004$). These two injection treatments showed marginally similar linear ($p=0.084$) and non-linear trends ($p=0.107$).

PCB Elimination

Lipid-normalized elimination rates and related statistics for PCBs are presented in Table 7. Concentration-time scatter plots of each PCB congener are presented in Figure 9. Linear regression techniques failed to detect significant ($p<0.05$) changes in PCB congener concentration with time. All correlation coefficients were low ($r^2<0.115$). Biological half-lives (BHL) and time to steady state (TSS) were not calculated because of the non-significance of the linear regression models, and these estimations would be extrapolations well beyond the time frame of this study.

PAH Elimination

Lipid-normalized elimination rates and related statistics for PAHs are presented in Table 8. Linear regression techniques detected significant ($p<0.05$) declines with time in phenanthrene, benz(*a*)anthracene, chrysene/triphenylene and benz(*g,h,i*)perylene. Marginally significant ($0.05<p<0.1$) linear regressions were detected with fluorene, anthracene, fluoranthene and pyrene. The regressions had a wide range of correlation coefficients, ranging from 0.009 in acenaphthene to 0.504 in benz(*a*)anthracene. Benz(*a*)pyrene concentrations declined below analytical detection limits before the first sampling date. Aside from benz(*a*)pyrene, benz(*g,h,i*)perylene had the highest elimination rate ($0.066 \pm 0.027\ d^{-1}$). Concentration-time scatter plots of each PAH congener are presented in Figure 10.

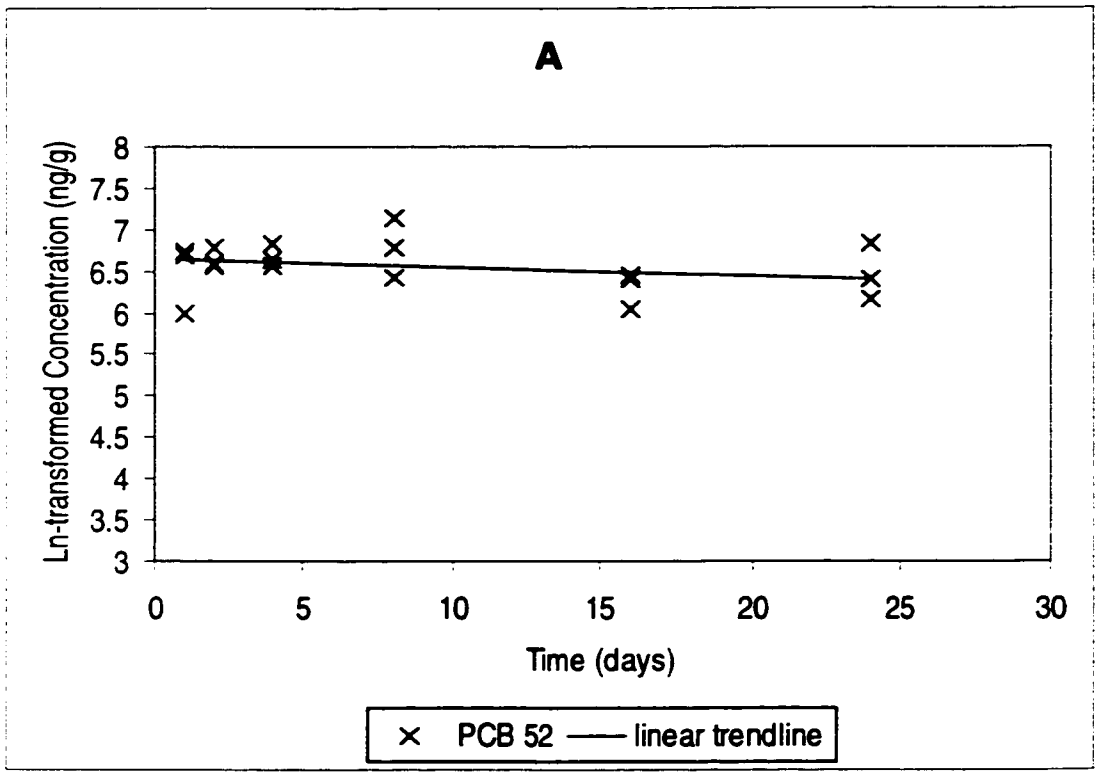
Table 6. Results of MANOVA#2, testing for trends in concentration over time and differences between control-corrected PCB and PAH injection treatments. Asterisks indicate significant ($p<0.05$) effects.

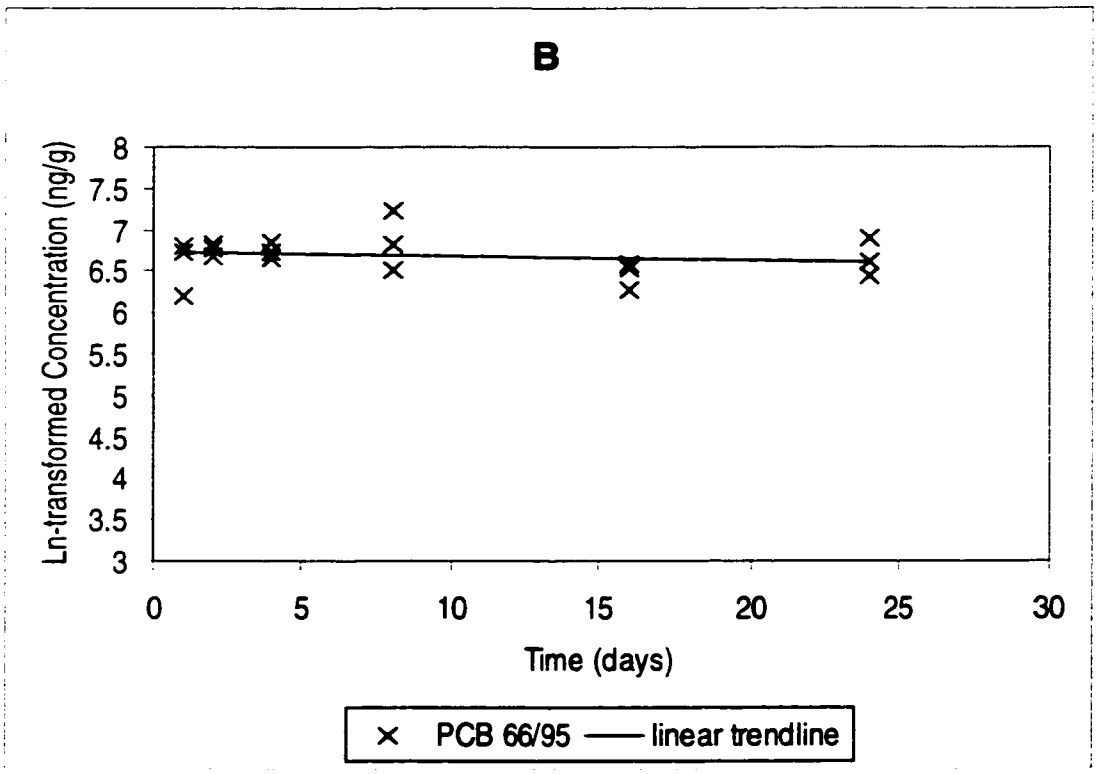
Test	Wilks' Lambda	F Value	Numerator Degrees of Freedom	Denominator Degrees of Freedom	Probability
Effect of **time** averaged across injection treatments:					
• Overall	0.001	2.22	40	24.59	0.02*
• Linear	0.102	5.51	8	5	0.04*
• Quadratic	0.252	1.85	8	5	0.26
Effect of **injection treatments** averaged across time:	0.0004	1419	8	5	0.0001*
Injection treatment x time **interaction**:					
• Overall	0.0006	2.82	40	24.59	0.004*
• Linear	0.146	3.67	8	5	0.084
• Quadratic	0.163	3.21	8	5	0.107

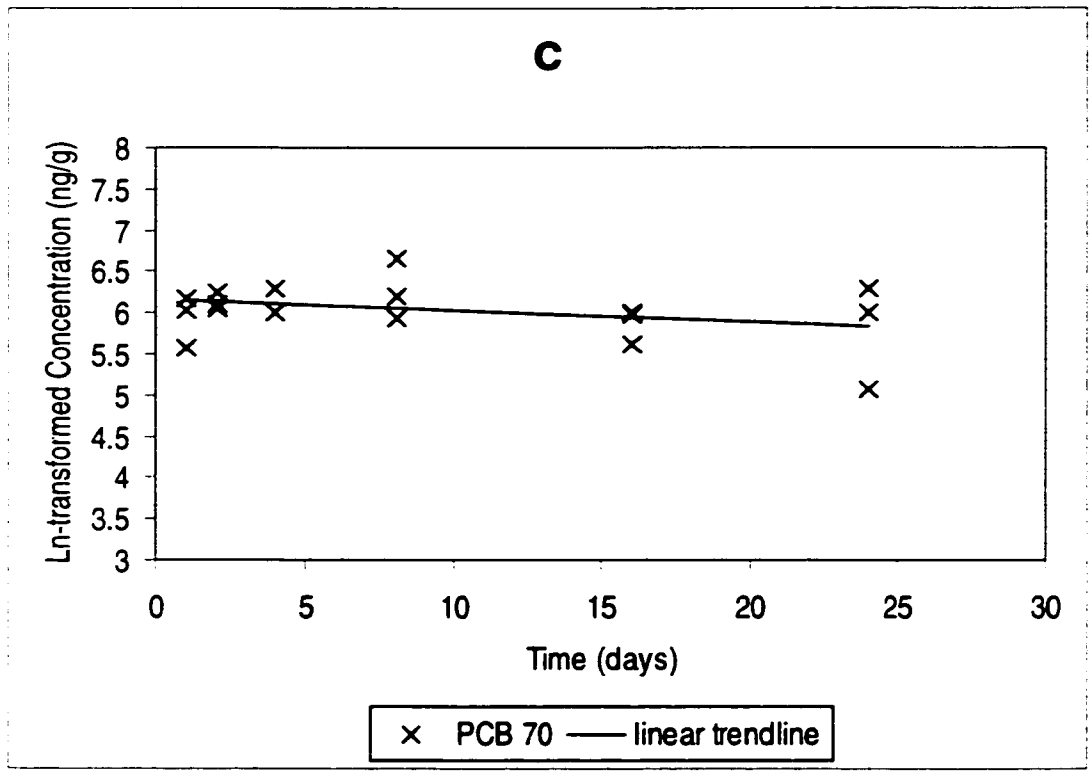
Table 7. PCB linear regression results and related statistics. Note that estimates of linear regression slopes were negative-transformed such that elimination rates are expressed as positive values.

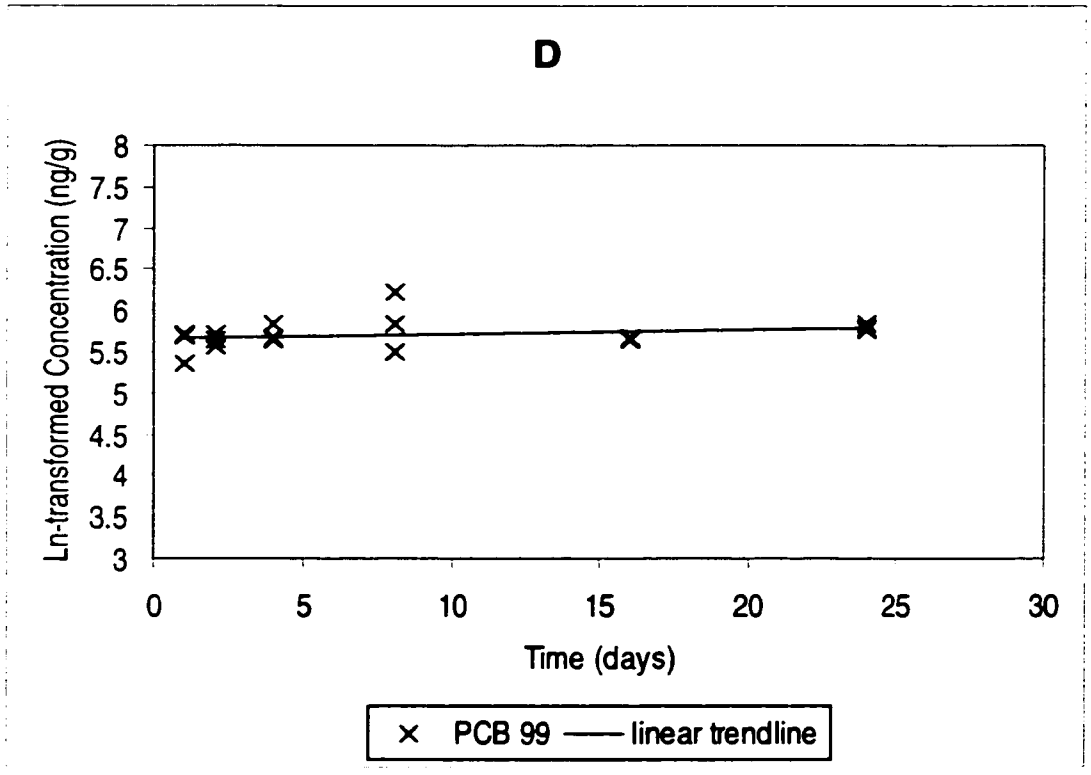
PCB Congener	Log K_{ow}	Elimination Rate (d^{-1})	Standard Error	Correlation Coefficient	Probability
52	5.84	0.009852	0.00827	0.0241	0.251
66/95	6.2	0.005066	0.00683	0.0332	0.4963
70	6.2	0.013633	0.00946	0.1148	0.169
99	6.39	-0.00515	0.00495	0.0633	0.3138
101	6.38	0.000587	0.00601	0.0006	0.9235
105	6.65	0.004259	0.00646	0.0265	0.5189
110	6.48	0.006612	0.0073	0.0487	0.3789
138	6.83	-0.002607	0.00478	0.0182	0.5932
149	6.67	0.002159	0.00612	0.0077	0.7291
153	6.92	-0.00496	0.00503	0.0572	0.3392
170/190	7.46	-0.00526	0.00497	0.0654	0.3058
174	7.11	-0.00133	0.00568	0.0034	0.8177
180	7.36	-0.004594	0.00505	0.0491	0.3769
182/187	7.2	-0.00498	0.00514	0.0555	0.3467
194	7.8	-0.00418	0.00551	0.0348	0.4589
206	8.09	-0.00506	0.00499	0.0605	0.3253

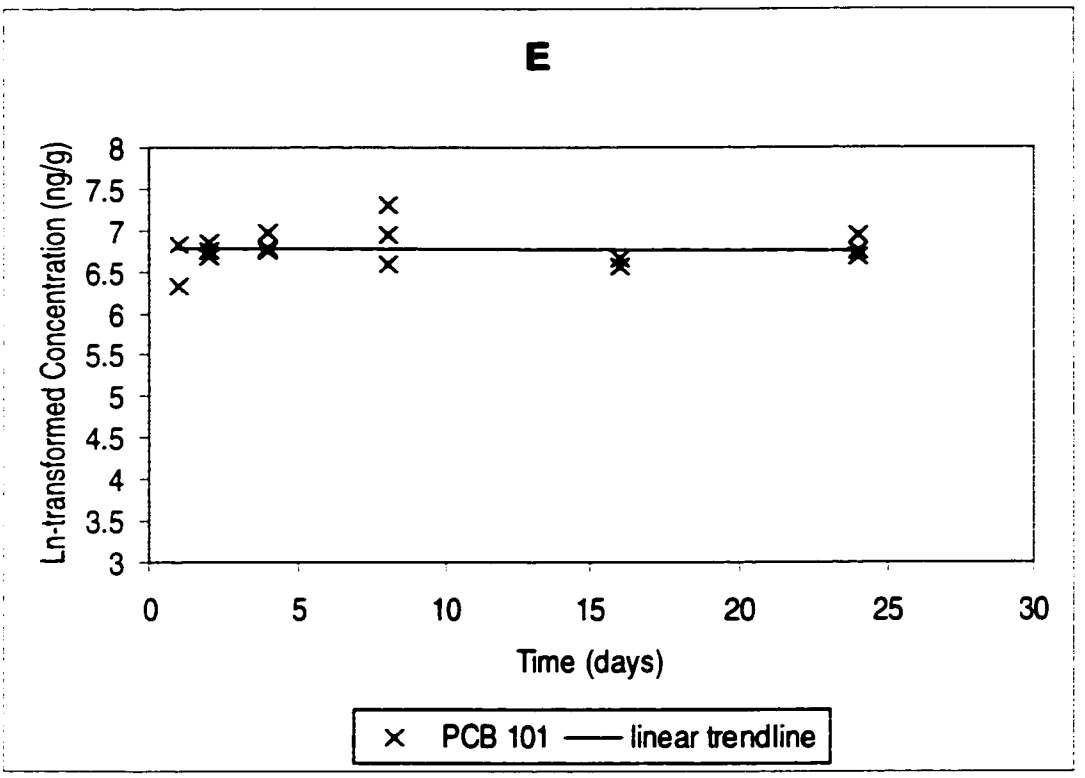
Figure 9. Concentration-time scatterplots of PCB congeners. A: PCB 52; B: PCB 66/95; C: PCB 70; D: PCB 99; E: PCB 101; F: PCB 105; G: PCB 110; H: PCB 138; I: PCB 149; J: PCB 153; K: PCB 170/190; L: PCB 174; M: PCB 180; N: PCB 182/187; O: PCB 194; P: PCB 206. Data points represent detection limit-corrected, lipid-normalized, ln-transformed congener concentrations of each replicate. Trendline represents line of best fit.

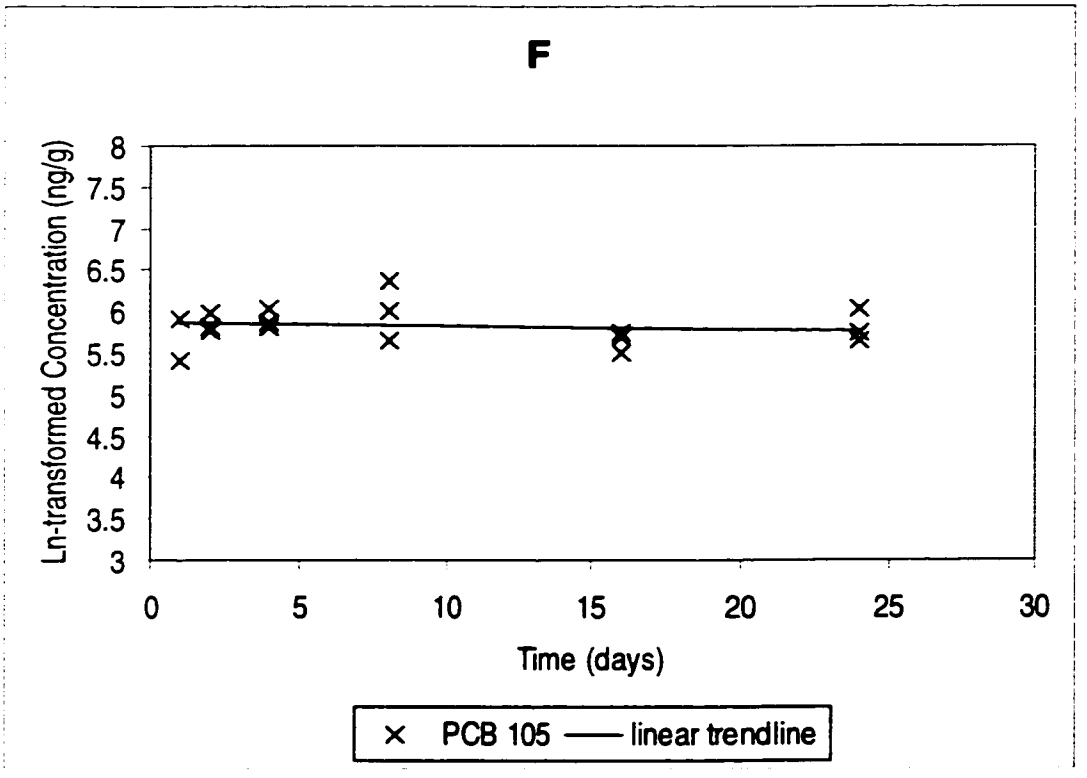

A
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 52
linear trendline

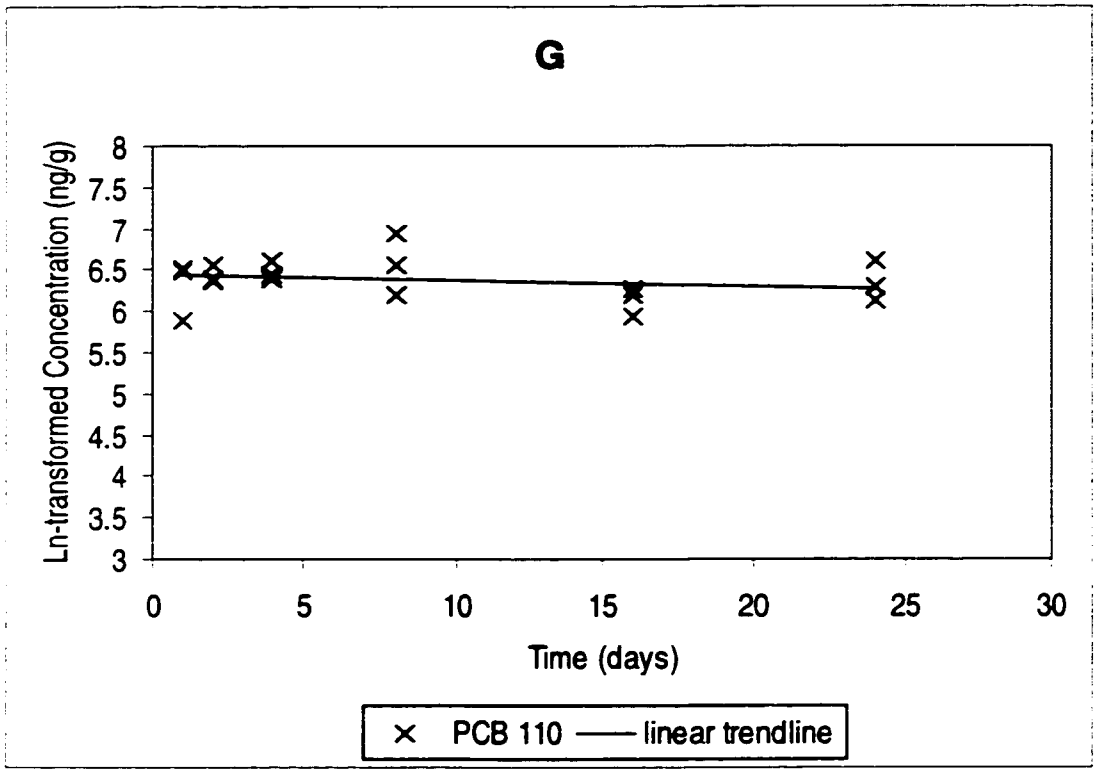

B
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 66/95
linear trendline

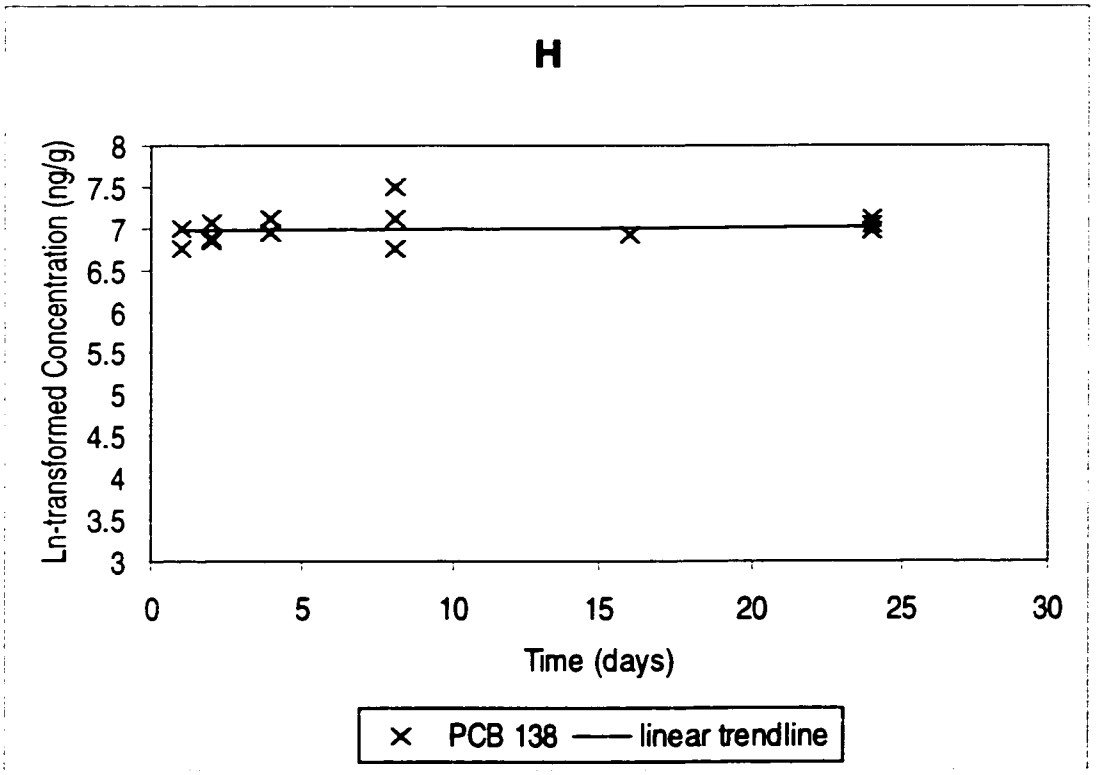

C
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 70
linear trendline


D
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 99
linear trendline

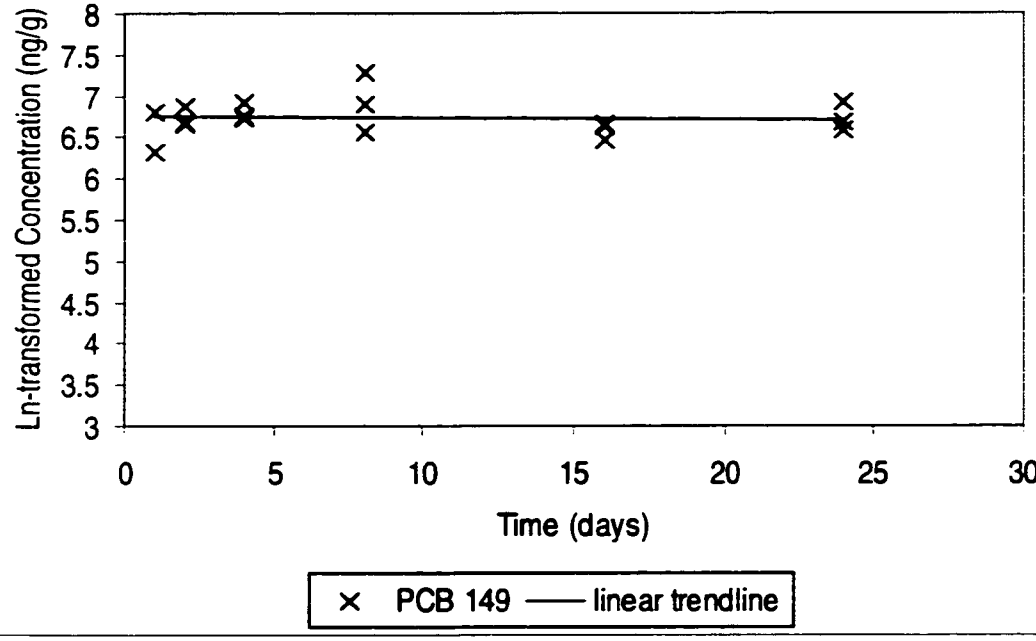

E
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 101
linear trendline


F
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 105
linear trendline

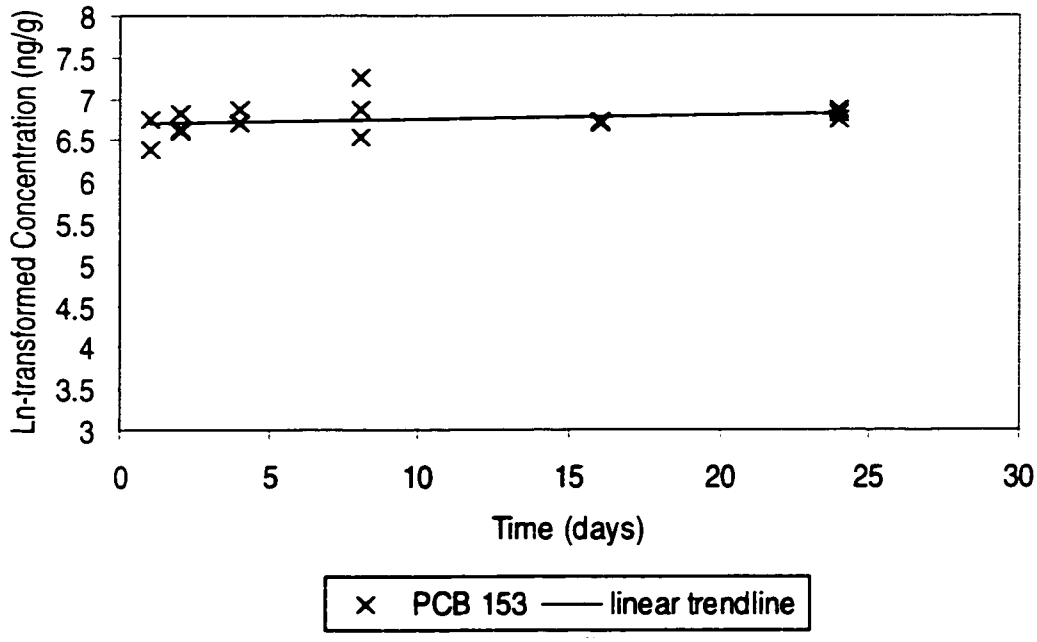

G
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 110
linear trendline

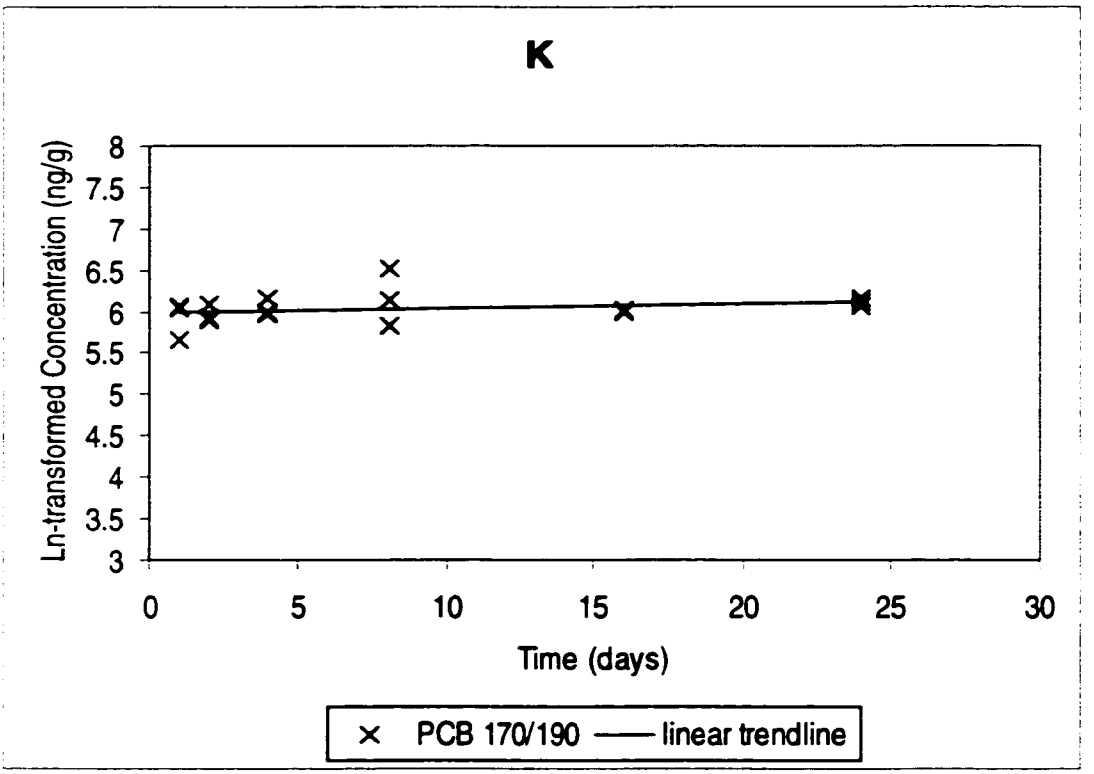

H
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 138
linear trendline

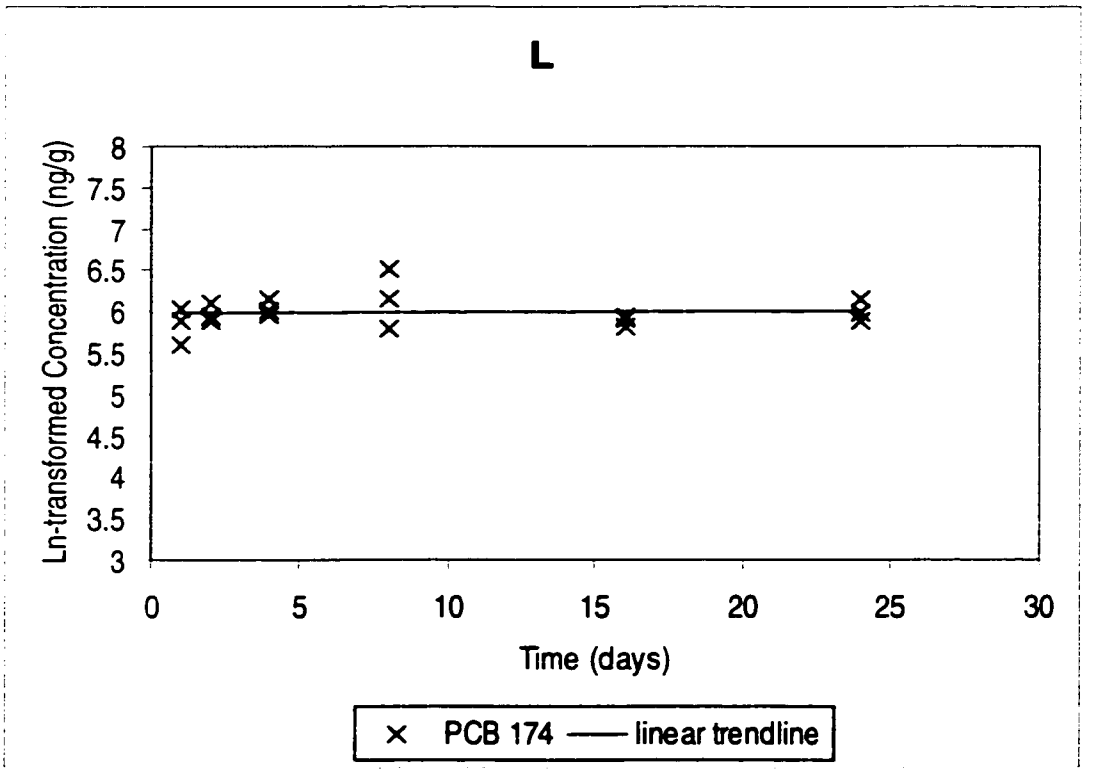
I


Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 149
linear trendline

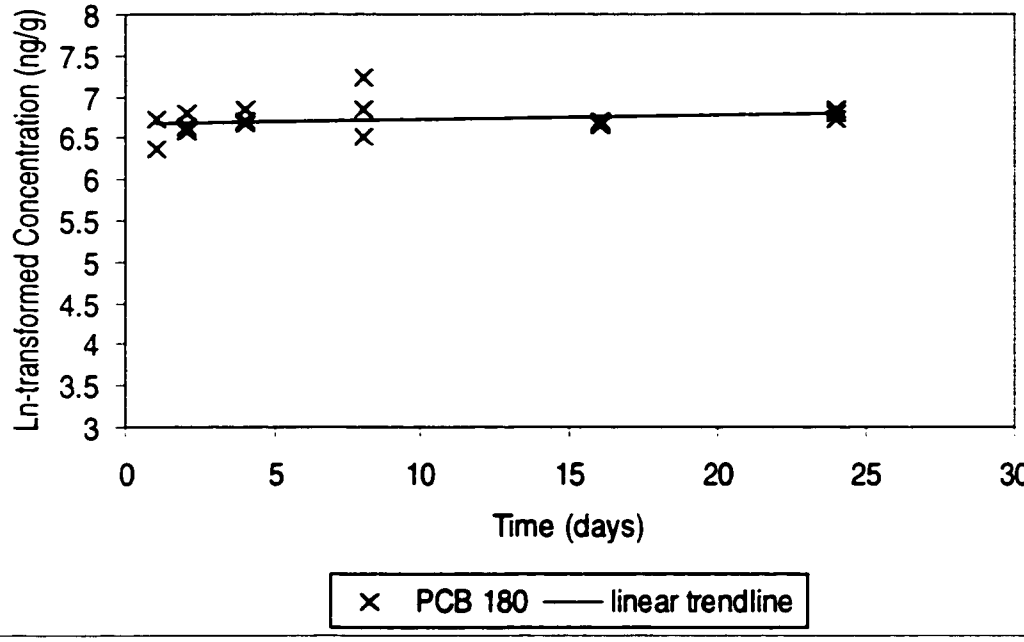
J


Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 153
linear trendline

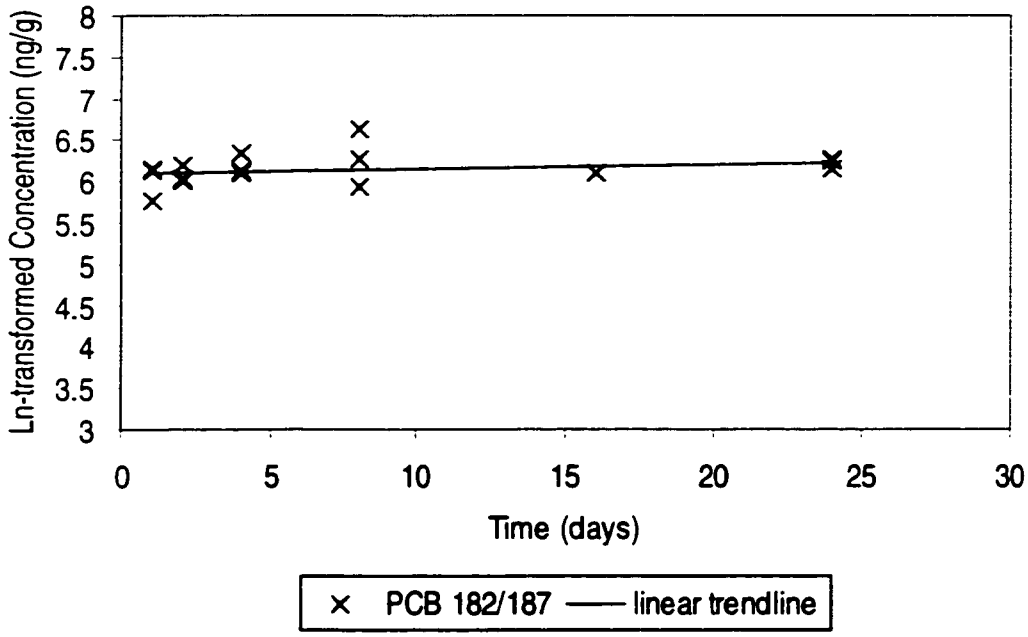

K
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 170/190
linear trendline

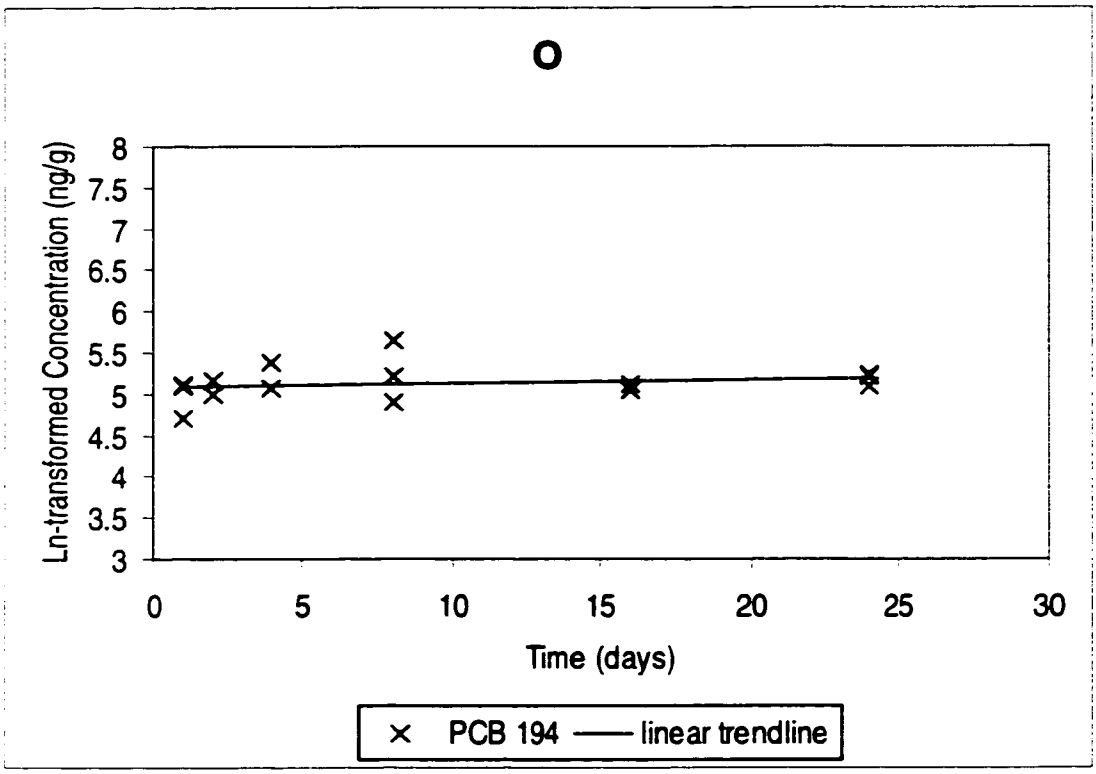

L
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 174
linear trendline

M


Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 180
linear trendline

N


Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 182/187
linear trendline


O
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 194
linear trendline

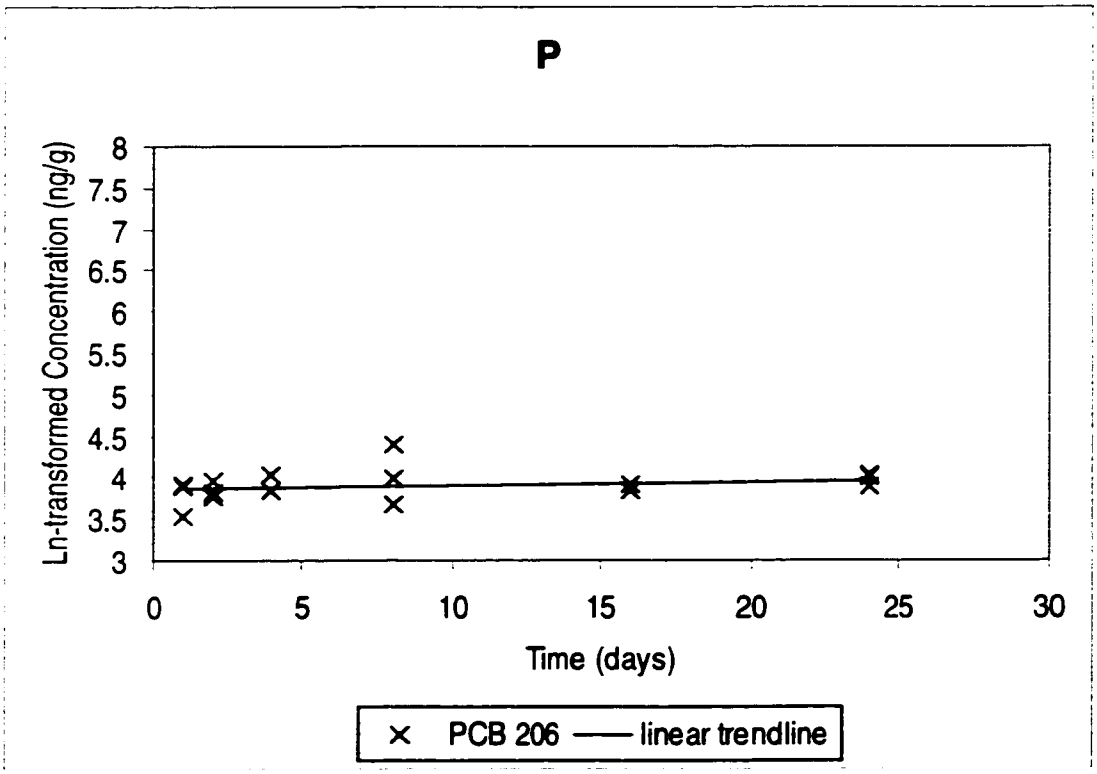
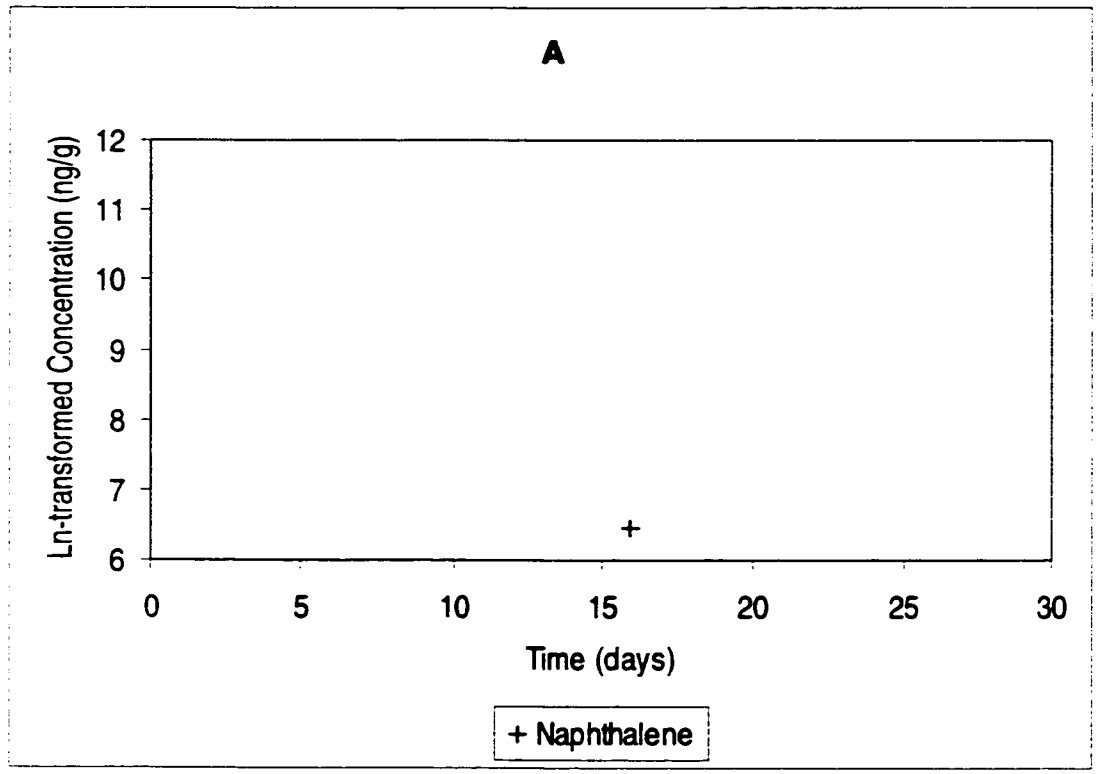
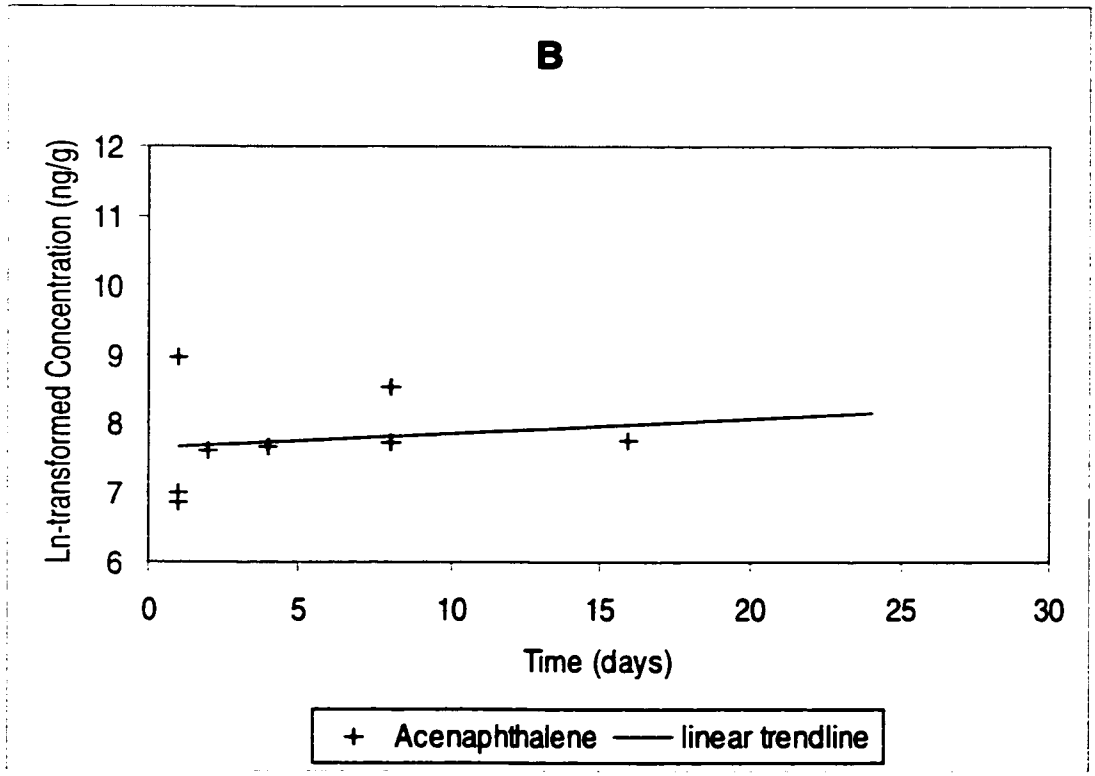

P
Ln-transformed Concentration (ng/g)
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
0
5
10
15
20
25
30
Time (days)
PCB 206
linear trendline

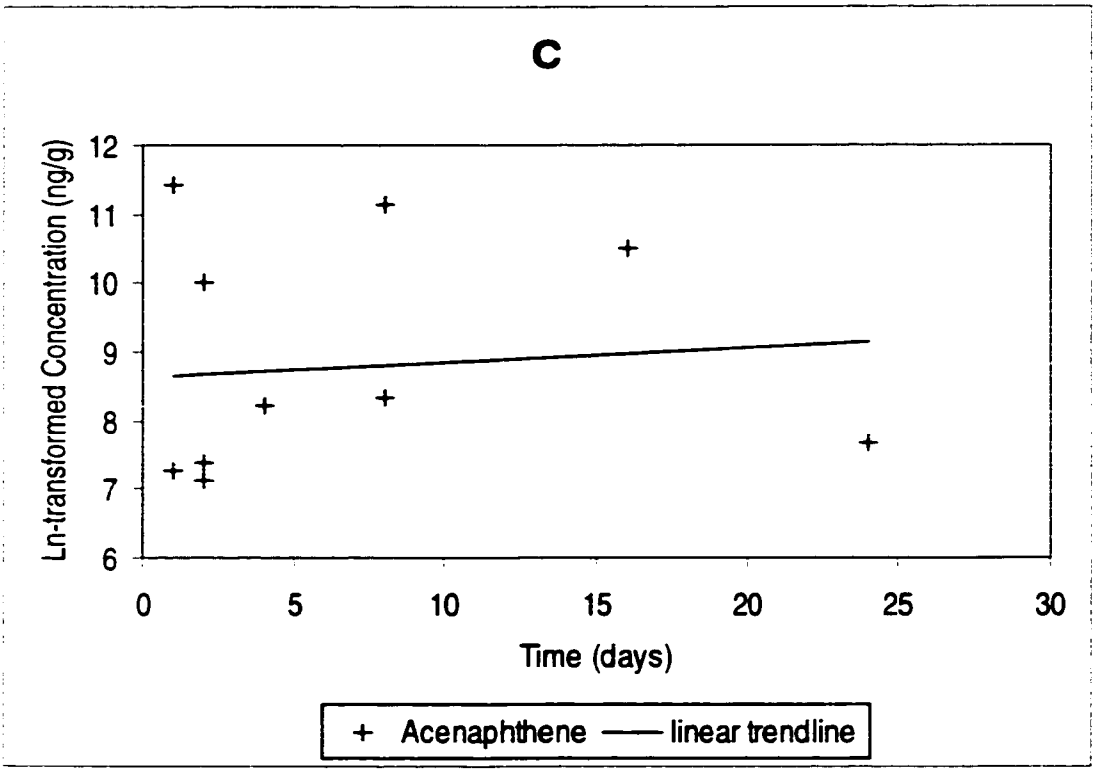
Table 8. PAH linear regression results and related statistics. Note that estimates of linear regression slopes were negative-transformed such that elimination rates are expressed as positive values. Asterisks indicate significance at the $p<0.1$ level. ND indicates where linear regression analyses could not be performed.

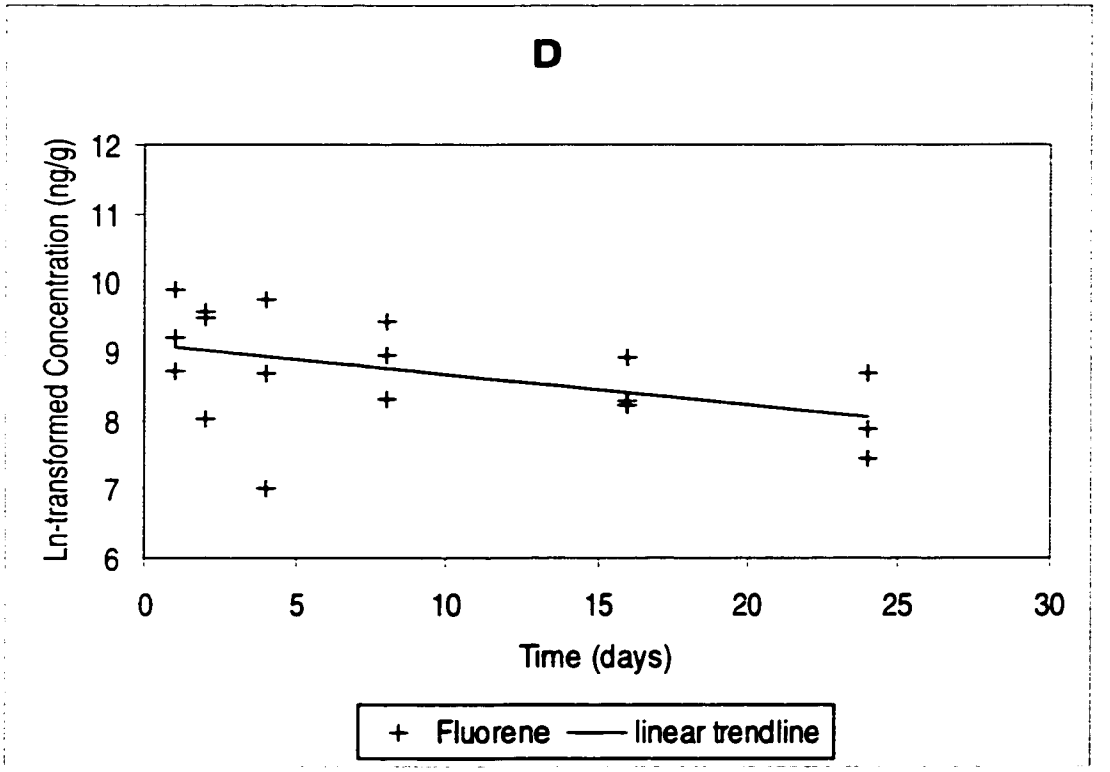
Congener	Log K_{ow}	Elimination Rate (d^{-1})	Standard Error	Correlation Coefficient	Probability
Naphthalene	3.37	ND	ND	ND	ND
Acenaphthylene	4	-0.021	0.053	0.026	0.7029
Acenaphthene	3.92	-0.021	0.074	0.009	0.7833
Fluoranthene	4.18	0.044	0.021	0.219	0.0504*
Phenanthrene	4.57	0.045	0.015	0.362	0.0106*
Anthracene	4.54	0.038	0.019	0.288	0.0719*
Fluoranthene	5.22	0.027	0.015	0.18	0.079*
Pyrene	5.18	0.032	0.018	0.163	0.0965*
Benz(*a*)anthracene	5.91	0.07	0.017	0.504	0.001*
Chrysene / Triphenylene	5.49	0.044	0.017	0.303	0.018*
Benz(*b*)fluoranthene	5.8	0.045	0.027	0.148	0.1155
Benz(*k*)fluoranthene	6	0.027	0.03	0.065	0.378
Benz(*a*)pyrene	6.04	ND	ND	ND	ND
Indeno(1,2,3-*c,d*) pyrene	6.5	0.01	0.016	0.021	0.5632
Dibenz(*a,h*)anthracene	6.75	-0.009	0.019	0.014	0.645
Benz(*g,h,i*)perylene	6.5	0.066	0.027	0.355	0.0316*

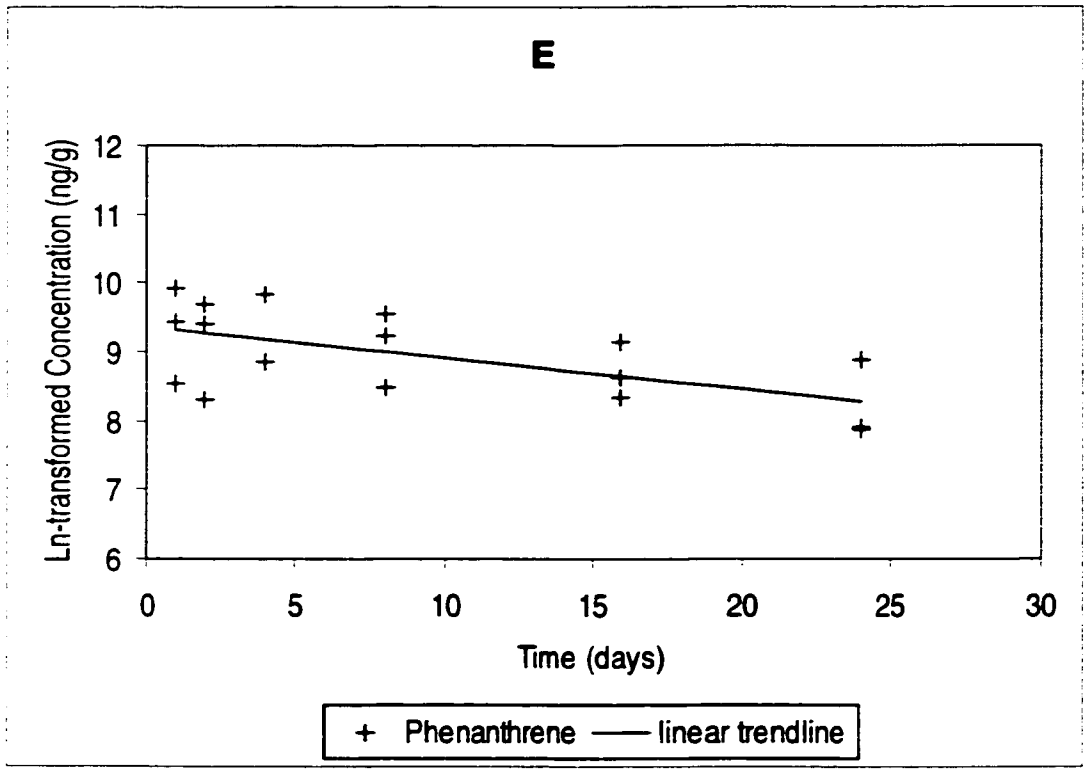
Figure 10. Concentration-time scatterplots of PAH congeners. A: Napphthalene; B: Acenaphthalene; C: Acenaphthene; D: Fuorene; E: Phenanthrene; F: Anthracene; G: Fluoranthene; H: Pyrene; I: Benz(*a*)anthracene; J: Chrysene / Triphenylene; K: Benz(*b*)fluoranthene; L: Benz(*k*)fluoranthene; M: Indeno(1,2,3-*c,d*)pyrene; N: Dibenz(*a,h*)anthracene; O: Benz(*g,h,i*)perylene. Scatterplot for benz(*a*)pyrene not included because all concentrations were below analytical detection limits. Data points represent lipid-normalized, detection-limit-corrected, ln-transformed congener concentrations of each replicate. Trendline represents line of best fit. Concentrations below analytical detection limits were not included in these graphs.

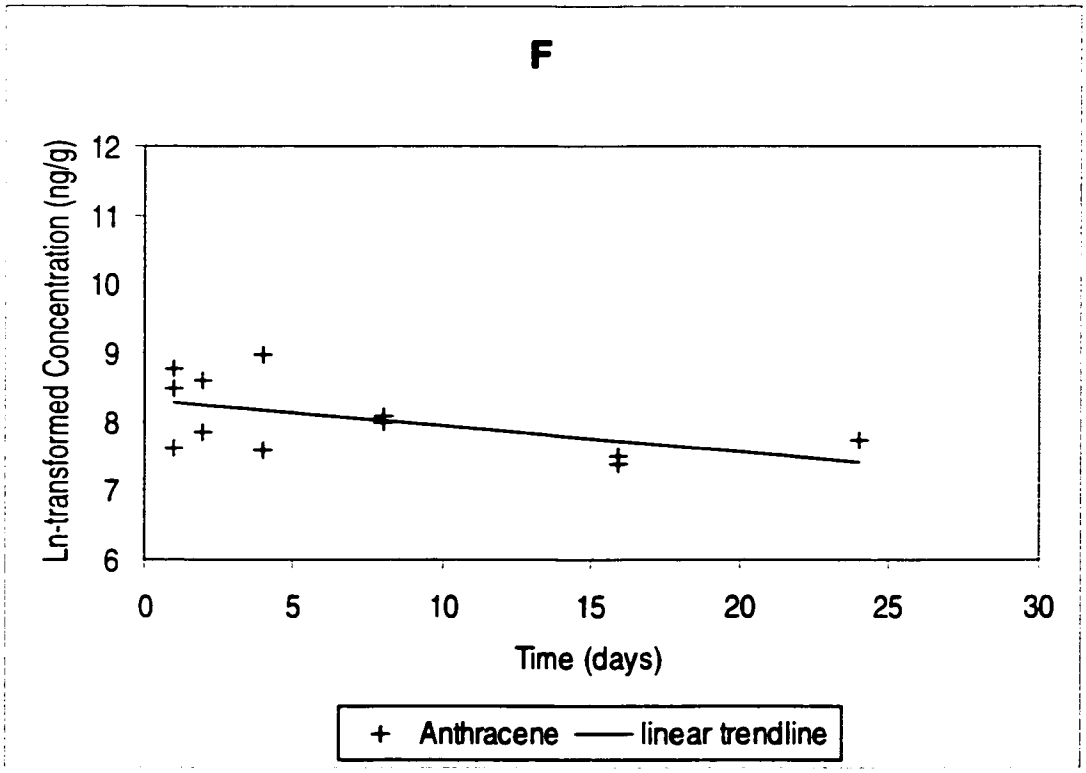

A
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Naphthalene

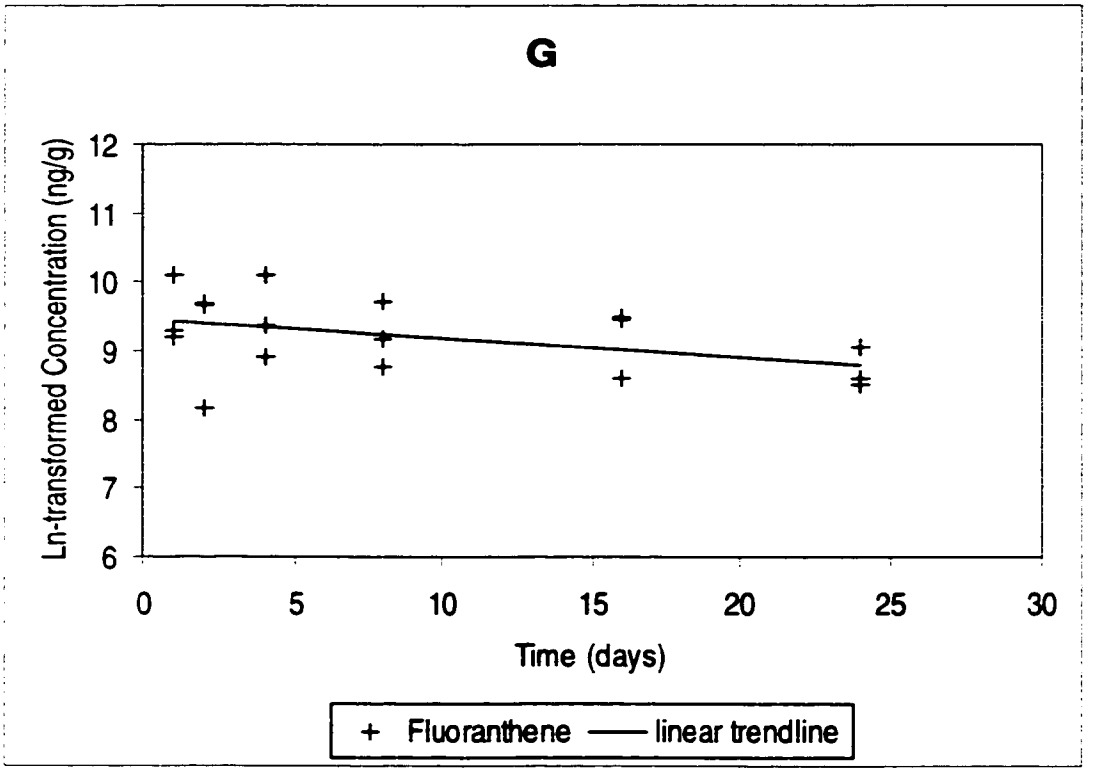

B
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Acenaphthalene
linear trendline

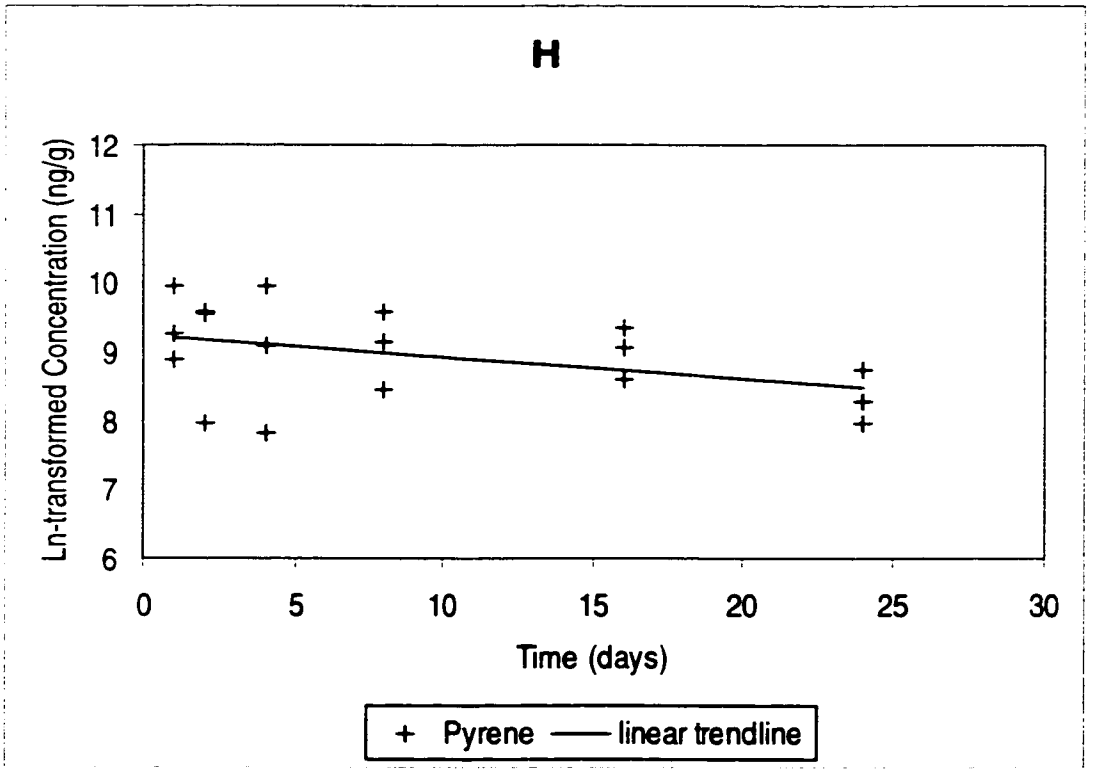

C
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Acenaphthene
linear trendline

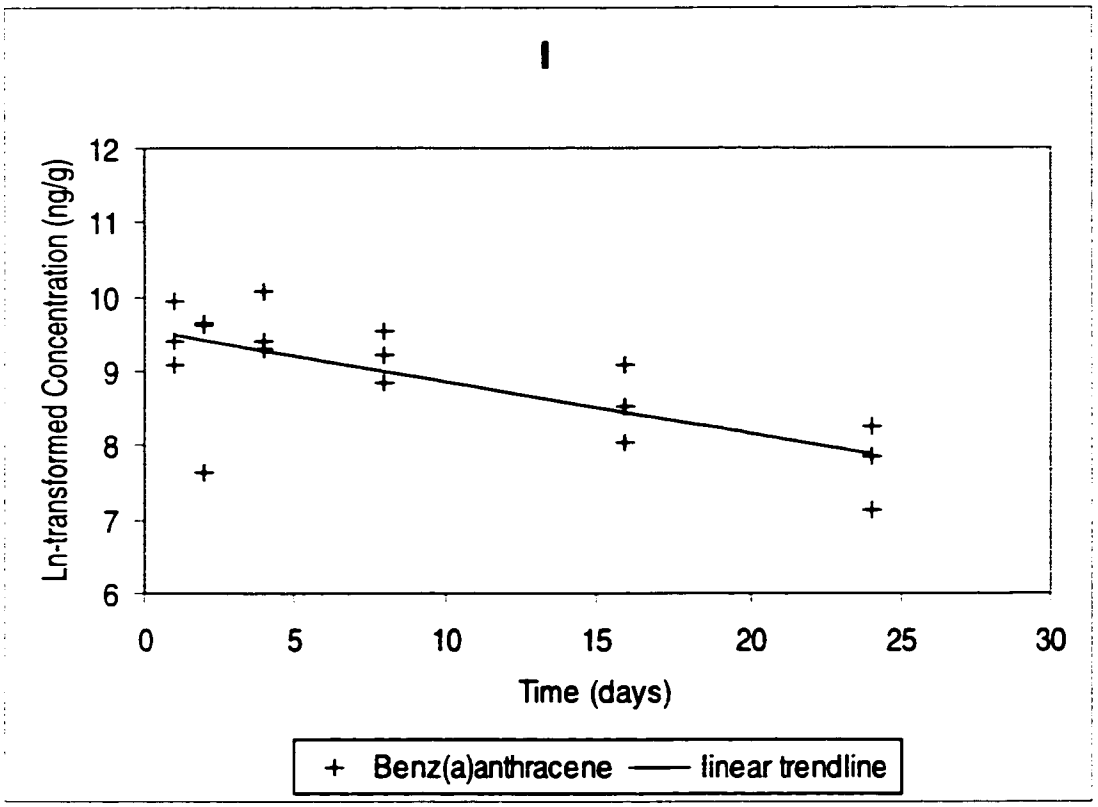

D
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Fluorene
linear trendline

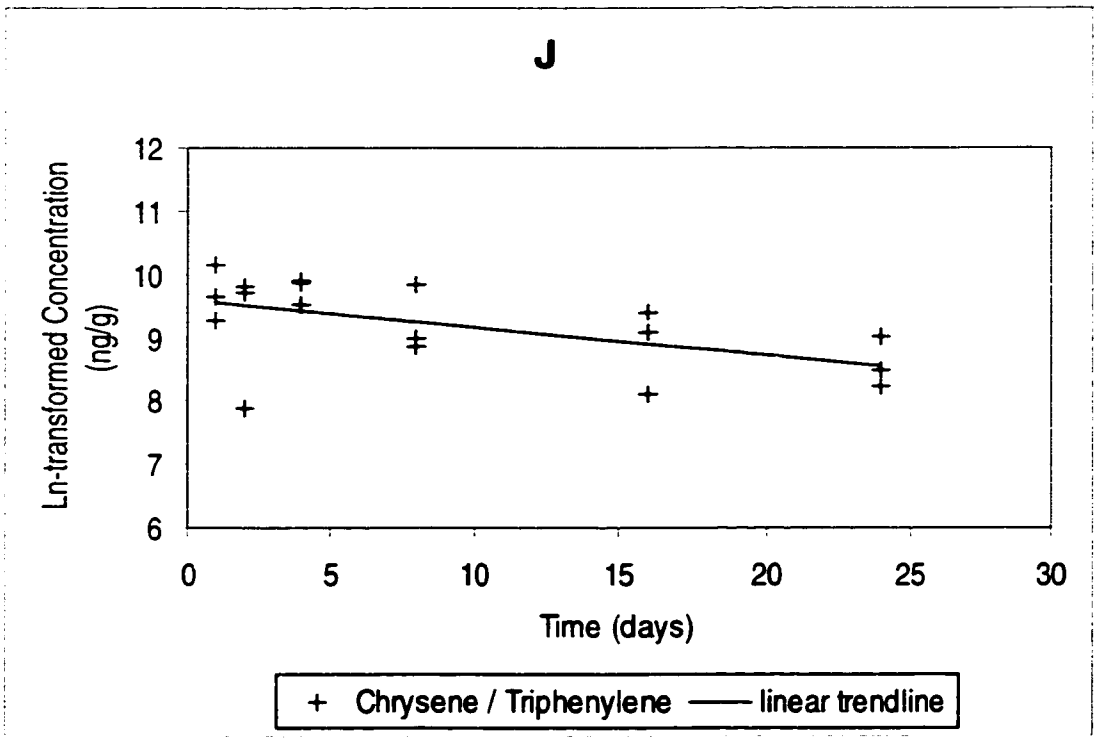

E
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Phenanthrene
linear trendline

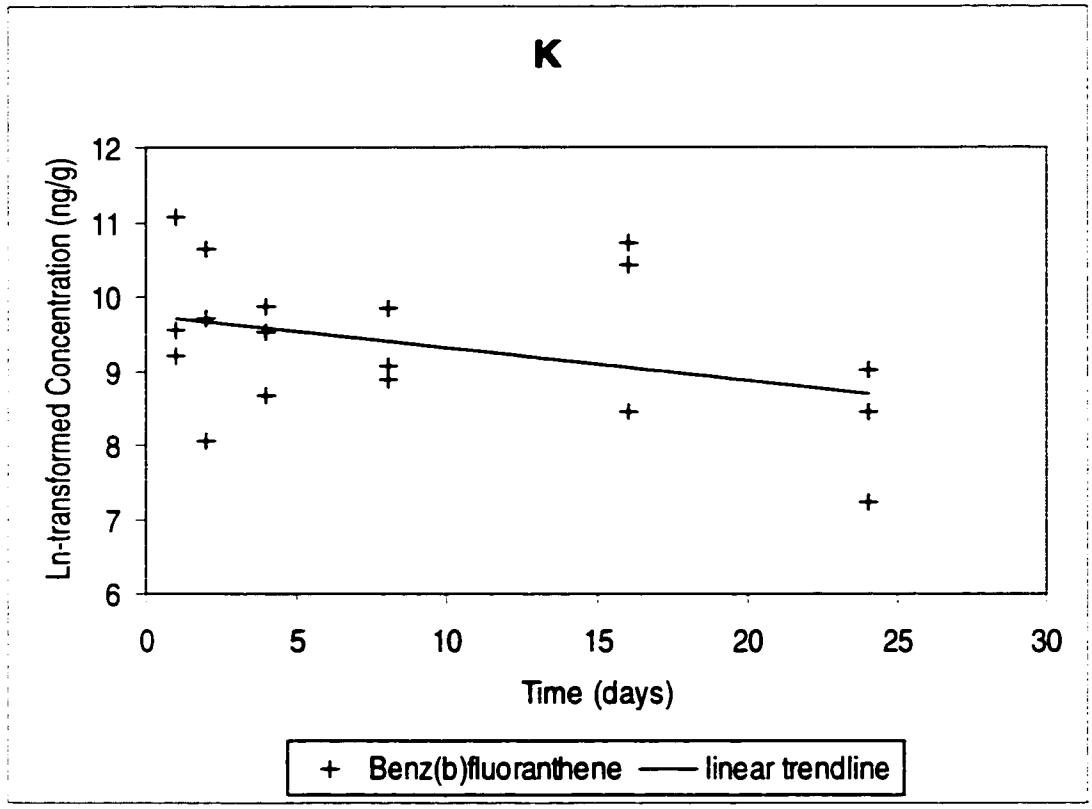

F
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Anthracene
linear trendline

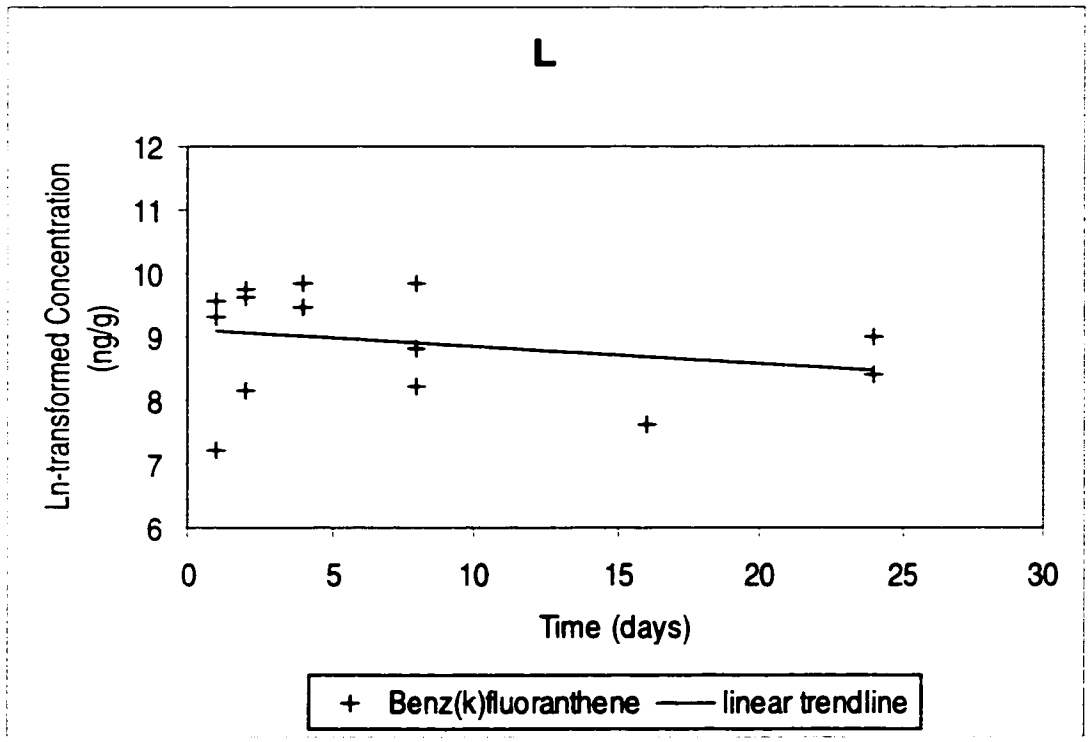

G
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Fluoranthene
linear trendline


H
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Pyrene
linear trendline

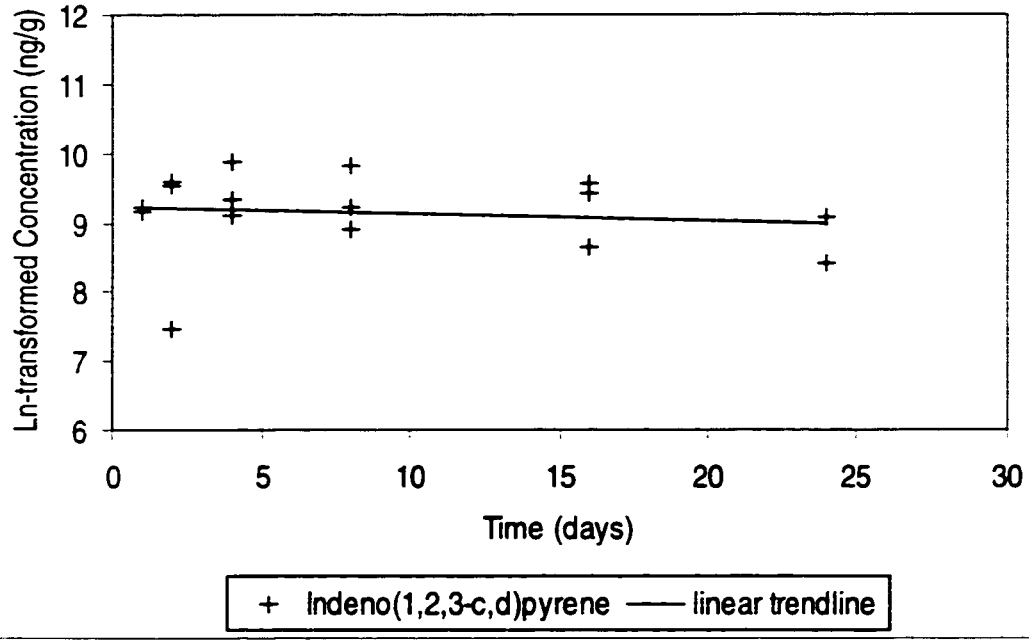

I
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Benz(a)anthracene
linear trendline


J
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Chrysene / Triphenylene
linear trendline

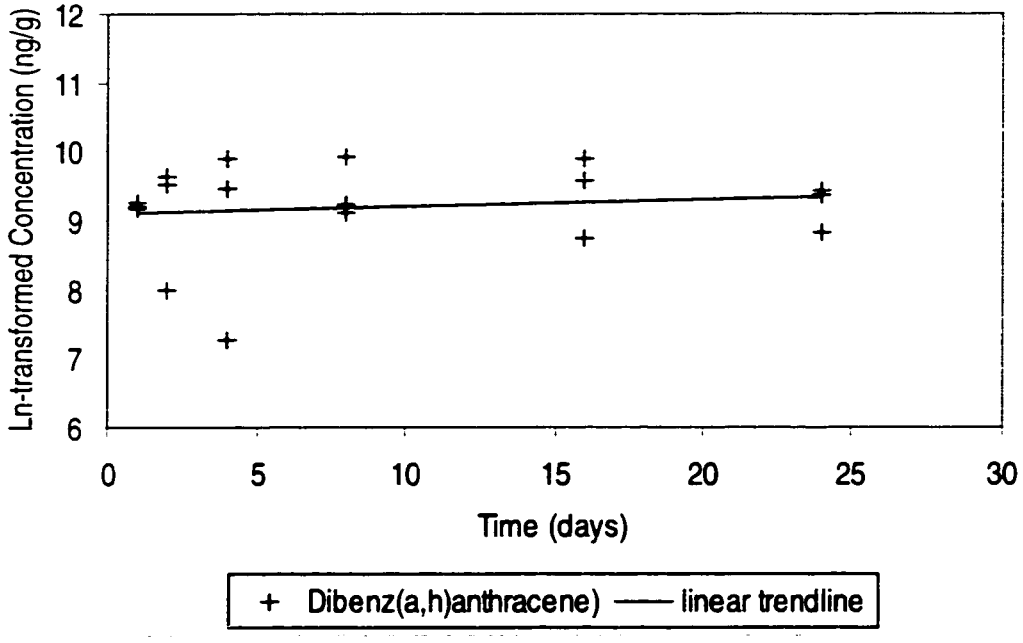

K
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Benz(b)fluoranthene
linear trendline

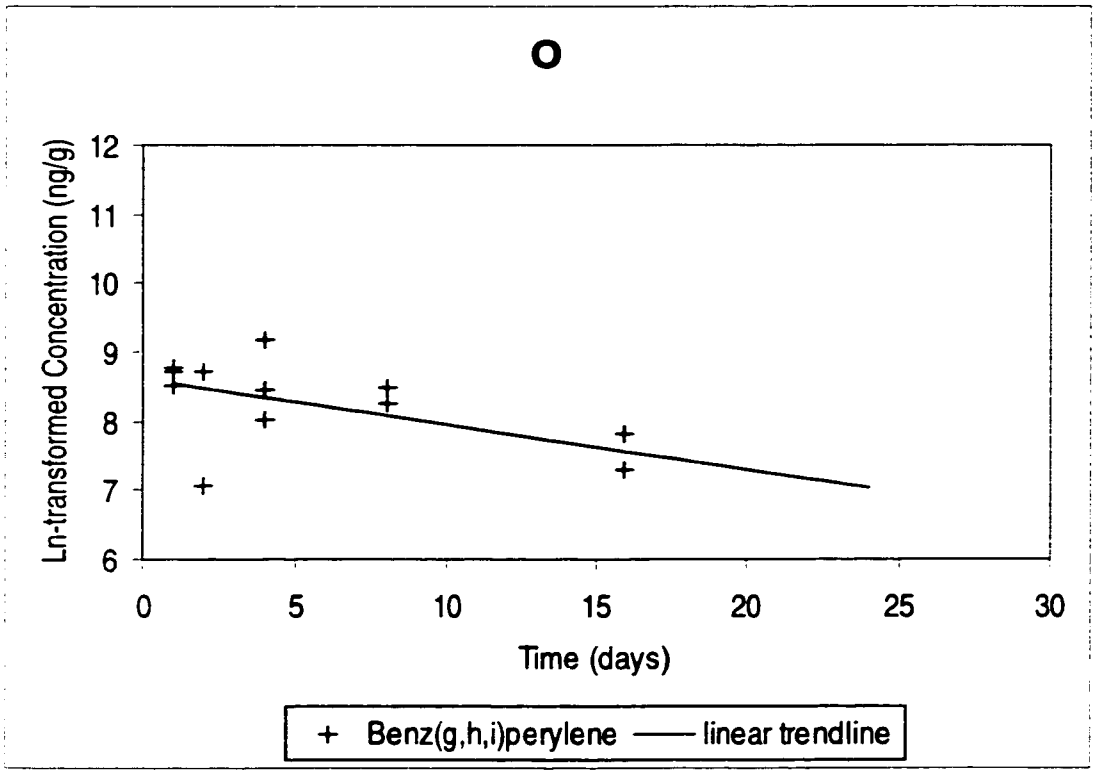

L
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
Benz(k)fluoranthene
linear trendline

M


Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Indeno(1,2,3-c,d)pyrene —— linear trendline

N


Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Dibenz(a,h)anthracene) —— linear trendline


o
Ln-transformed Concentration (ng/g)
12
11
10
9
8
7
6
0
5
10
15
20
25
30
Time (days)
+ Benz(g,h,i)perylene
linear trendline

The biological half-lives for the PAH congeners that were significantly eliminated (or marginally so) are presented in Table 9. Biological half-lives ranged from approximately 10 days (benz(*a*)anthracene to 25.6 days (fluoranthene). Times to steady state were not calculated, as these estimations would be extrapolations well beyond the time frame of this study.

PCB Elimination vs. Hydrophobicity

A relationship between PCB elimination rate and hydrophobicity (log K_{ow}) could not be determined in this study due to the non-significance of the ln-transformed PCB concentration vs. time linear regressions from which elimination rates are derived.

PAH Elimination vs. Hydrophobicity

Using the elimination rates estimated from the statistically significant ($p<0.1$) ln-transformed PAH concentration vs. time, linear regression did not detect a significant relationship with log K_{ow} ($p=0.104$, $r^2=0.38$) (Table 10); however, the probability was marginal, and there appeared to be a trend of increasing elimination rate with increasing hydrophobicity. The elimination rate – log K_{ow} scatterplot for PAHs is presented in Figure 11.

To summarize, no significant elimination of PCBs took place in the time frame used for this study. Significant elimination occurred in several PAHs. Naphthalene and benz(*a*)pyrene concentrations declined below analytical detection limits within 24 hours of injection. PAH elimination rates seemed to increase with increasing hydrophobicity; however, the relationship was not statistically significant.

Table 9. Statistically significant ($p<0.1$) PAH elimination rates and estimates of biological half lives.

PAH Congener	Elimination Rate (d^{-1})	Biological Half-Life (days)
Fluorene	0.044	15.7
Phenanthrene	0.045	15.4
Anthracene	0.038	18.2
Fluoranthene	0.027	25.7
Pyrene	0.032	21.7
Benz(a)anthracene	0.07	9.90
Chrysene/Triphenylene	0.044	15.8
Benz(g,h,i)perylene	0.066	10.5

Table 10. Elimination rate – log K_{ow} regression results using PAH elimination rates derived from statistically significant ($p<0.1$) ln-transformed concentration vs. time linear regressions.

Correlation Coefficient	Slope	Standard Error (slope)	Intercept	Standard Error (intercept)	F Value	Probability
0.38	0.012	0.006	-0.017	0.033	3.67	0.104

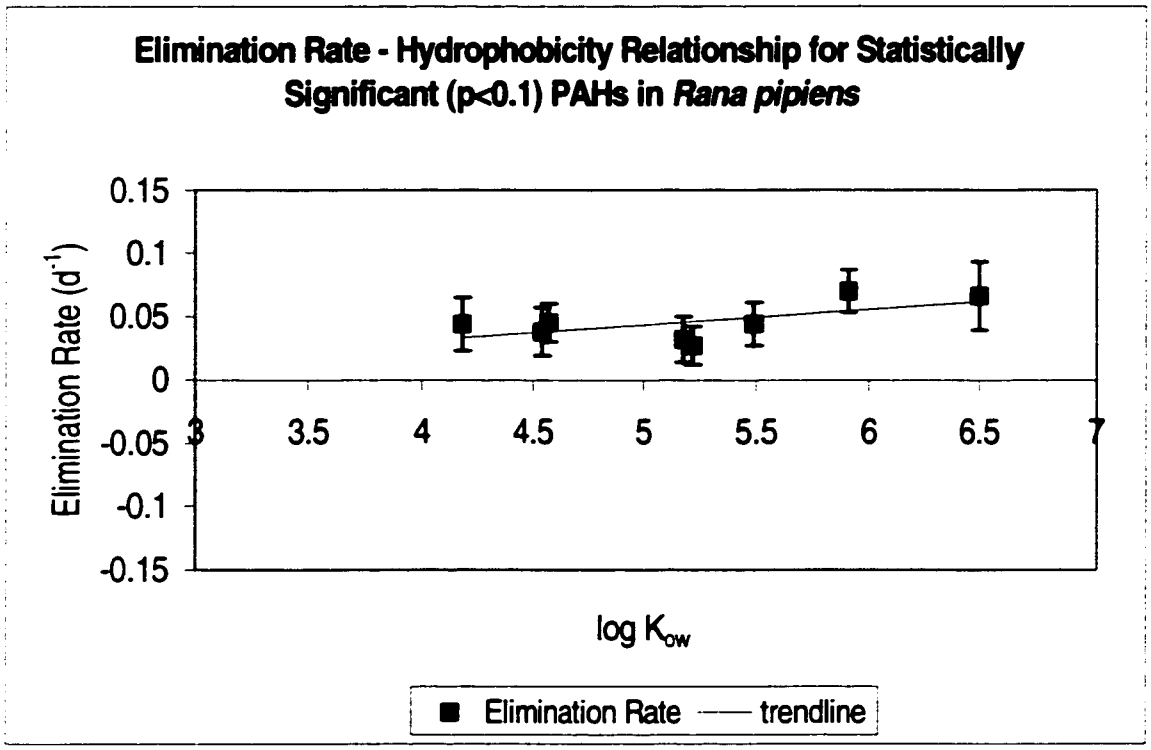


Figure 11. Elimination – log K_{ow} scatter plots of PAHs. Data points represent elimination rates determined by statistically significant ($p<0.1$) linear regressions of individual congeners. Error bars represent standard errors of elimination rates.

Discussion

The results of this elimination study indicate that no significant PCB elimination occurred during the 24-day course of the experiment. The elimination period was too short to detect any PCB elimination and quantify statistically meaningful elimination rates. The extremely slow elimination of PCBs from the frogs used in this study lends credence to the biological persistence of PCBs in amphibians and their potential to cause narcosis in these organisms, which seem to be unable to biodegrade them.

However, several PAH congeners were rapidly eliminated, with concentrations in some of the frogs falling below analytical detection limits early in the experimental time frame. No benz(*a*)pyrene was detected 24 hours after injection, and naphthalene was detected in only one of the PAH-treated frogs. Concentrations of acenaphthene, acenaphthalene, phenanthrene, anthracene, benz(*k*)fluoranthene and benz(*g,h,i*)perylene also fell below analytical detection limits in some of the frogs. The rapid elimination of certain PAH congeners from some or all of the frogs, as well as the lack of a significant relationship between PAH elimination rate and hydrophobicity (log K_{ow}) suggests that some PAHs were being metabolized, and may therefore pose a genotoxic threat to amphibian communities exposed to these contaminants.

Experimental Design MANOVAs

Results of MANOVA#2 indicated that analyte concentrations declined over time, suggesting that averaged across the injection treatments, elimination was taking place. Relative PCB and PAH analyte concentrations differed, reflecting the difference in dosage between the PCB and PAH injection treatments. Concentration trends over time differed between PCB and PAH injection treatments, indicating that PCBs behaved differently than PAHs in the frogs used in this study. The marginal similarity between linear and non-linear PCB and PAH concentration-time trends, however, may be an artifact of grouping the PCB and PAH congeners to make the MANOVA analysis possible.

1a) Is PCB Elimination Influenced by Hydrophobicity?

Linear regression analyses failed to detect a significant change in PCB congener concentration over time. Scatterplots of PCB congener concentration – time show that PCB congener concentration changed very little over the course of the study (see Figure 9). Indeed, the linear regression estimates of slope (elimination rate) were not significantly different from zero. The low linear regression correlation coefficients support that the experimental time frame (24 days) was too short for any statistically significant PCB elimination to take place. The findings of this study suggest that PCBs with four chlorine atoms or more are persistent in adult amphibians.

The biological half-lives (BHL) (calculated from elimination rate) of various PCBs determined from a variety of organisms further emphasizes the biological persistence of PCBs, especially as the degree of chlorination increases. BHL ranged from 12.8 days (pentachlorinated - PCB 101) to 28.9 days (hexachlorinated - PCB 156) in earthworms (*Eisenia andrei*) (Belfroid et al. 1995); 4 days (tetrachlorinated - PCB 64) to 16.5 days (heptachlorinated - PCB 183) in zebra mussels (*Dreissena polymorpha*) (Morrison et al. 1995); 3.7 days (tetrachlorinated - PCB 57) to 11.7 days (octachlorinated - PCB 202) in green-lipped mussels (*Perna viridis*) (Tanabe et al. 1987); 0.7 days (dichlorinated - PCB 9) to over 11000 days (decachlorinated - PCB 209) in zebrafish (*Brachydanio rerio*) (Fox et al. 1994); 5 days (dichlorinated - PCB 11) to over 1000 days for pentachlorobiphenyls in rainbow trout (*Oncorhynchus mykiss*) (Niimi and Oliver 1983); 47.2 days (tetrachlorinated - PCB 77) to 108.3 days (hexachlorinated - PCB 169) in rainbow trout (Coristine et al. 1996); and 28.3 days (trichlorinated - PCB 18) to 106.6 days (hexachlorinated - PCB 153) in juvenile rainbow trout (Fisk et al. 1998). The BHL of PCBs seem to be species-specific, and the BHL differences between species may reflect the ability to biodegrade this group of persistent pollutants. The comparability of these BHL, however, is limited due to the varying experimental conditions under which these studies were conducted.

Using the elimination rates of PCBs in various organisms presented in previously published studies, scatterplots against log K_{ow} generally show that elimination rates decline with increasing hydrophobicity (Appendix 1). In the earthworm study conducted by Belfroid et al. (1995), elimination rates declined with increasing hydrophobicity

(Appendix 1a). In zebra mussels, PCB elimination rates also decreased with increasing log K_{ow} (Appendix 1b) (Morrison et al. 1995). Green-lipped mussels also showed a similar trend (Appendix 1c) (Tanabe et al. 1987). In zebrafish, elimination rates seem to decline exponentially as hydrophobicity increases (Appendix 1d) (Fox et al. 1994). Using the elimination rates from the work of Coristine et al. (1996), PCB elimination rates also decline with increasing hydrophobicity in adult rainbow trout (Appendix 1e). A similar trend was observed with juvenile rainbow trout (Fisk et al. 1998) (Appendix 1f). Linear regression analyses indicate marginally significant to significant linear relationships between elimination rates and log K_{ow} in the above studies (Table 11). By far, zebrafish had the steepest elimination rate – hydrophobicity relationship, followed by zebra mussel > green-lipped mussel > earthworm > adult rainbow trout > juvenile rainbow trout. Because of differences in hydrophobicities, different PCB congeners are cleared from animal tissues at different rates, and the same congeners may be cleared at different rates by different organisms (Matthews and Dedrick 1984). The comparability of these relationships, however, is limited due to the varying experimental conditions under which these studies were conducted.

The one-compartment model used in this study may be inappropriate for adult amphibians. This model describes the simplest scenario, where uptake and elimination of hydrophobic compounds occurs through hydrophobicity-governed passive diffusion to and from water, and thus apply to aquatic organisms that are able to eliminate hydrophobic contaminants into ambient water (Walker 1987; Barron et al. 1990). Compartmental models should be based on the physiology and biochemistry of the system being modeled (Barron et al. 1990). Adult amphibians pose an interesting modeling problem: they are intermediate between aquatic and terrestrial, and this is reflected in their life history characteristics and physiologies.

Because of the terrestrial nature of adult amphibians, they are not always surrounded by water to which hydrophobic organic contaminants could be eliminated. Amphibians have permeable skin, but elimination of hydrophobic compounds by this route would be restricted by volatility. In terrestrial organisms, excretion of strongly hydrophobic compounds via bile or urine is minimal, and elimination of such compounds is reliant on their conversion into water-soluble metabolites (Walker 1987). The

Table 11. PCB elimination rates vs. log K_{ow} relationships from other studies. Earthworm (Belfroid et al. 1995); zebra mussel (Morrison et al. 1995); green-lipped mussel (Tanabe et al. 1987); zebra fish (Fox et al. 1994); rainbow trout (Coristine et al. 1996); and juvenile rainbow trout (Fisk et al. 1998). Asterisks indicate significance at the $p<0.05$ level.

	Earth-worm	Zebra Mussel	Green-lipped Mussel	Zebra Fish	Rainbow Trout	Juvenile Rainbow Trout
Slope	-0.029	-0.040	-0.031	-0.131	-0.003	-0.002
Standard Error (slope)	0.007	0.005	0.007	0.035	0.002	0.001
Intercept	0.235	0.354	0.312	0.931	0.032	0.028
Standard Error (intercept)	0.047	0.034	0.048	0.235	0.012	0.008
Correlation Coefficient	0.787	0.696	0.328	0.346	0.254	0.226
F value	18.43	66.23	17.57	13.76	3.74	4.08
Probability	0.0078*	0.0001*	0.0002*	0.001*	0.0791	0.063

persistence of all the PCBs analyzed in the frogs used in this study, regardless of log K_{ow}, suggest that adult amphibians may be unable to convert PCBs with 4 or more chlorine atoms into water-soluble metabolites that can be eliminated.

The results of the PCB elimination study indicate that the PCBs used in this study are remarkably persistent in adult leopard frogs. If this holds true for other amphibian species, the role as both predator and prey puts this taxon at risk of narcosis and reproductive failure from dietary accumulation of PCBs, and may endanger their predators by passing these persistent pollutants to the next trophic level. Russell et al. (1995b) found that biomagnification occurred only for chemicals with a log Kow of greater than 6.1. A study conducted by Niethammer et al. (1984) illustrated the biomagnification of PCBs and other chlorinated pollutants with increasing trophic level in a Louisiana watershed. PCB bioaccumulation factors increased with trophic level in a freshwater stream ecosystem (Hill and Napolitano 1997), and in a Massachusetts estuary (Lake et al. 1995). PCB concentrations increased with increasing trophic level (plankton to sculpins) in a Lake Michigan food chain (Evans et al. 1991). Thomann (1981) concluded that in aquatic food chains, most PCB residues in top predators could be attributed to PCB transfer from contaminated food. Zimmerman et al. (1997) found that fish-eating birds exhibited higher PCB body burdens than mussel-feeding ducks, and concluded that these species differences were due to the prey fish belonging to a higher trophic level than mussels. PCB contamination of food sources was assumed to be responsible for the impaired reproduction and resultant extirpation of the European otter (*Lutra lutra*) (a top predator) from Switzerland (Mason and Ratford 1994). PCB body burdens in wild fowl may be sufficiently high to produce concentrations in eggs that could endanger their young, and PCBs present in lactating animals can be transferred to nursing young (Matthews and Dedrick 1984).

Adult amphibians may prove to be excellent biomonitors of PCB congeners because of their inability to appreciably eliminate these chemicals. Adult amphibians, however, should not be used to track temporal fluctuations in environmental PCB concentrations because of their slow PCB kinetics. In order to use amphibians as monitors of PCB concentrations, further studies of substantially longer time frame need to be conducted in order to determine uptake and elimination rate constants to an

appropriate degree of statistical significance. In addition, uptake and elimination models need to be developed to reflect the unique physiology of adult amphibians. This is required in order to calibrate adult amphibians as biomonitors, and to determine if there is a relationship between physico-chemical properties (ie. K_{ow}) and biological persistence (e.g. elimination rate).

1b) Is PAH Elimination Influenced by Hydrophobicity?

Linear regression analyses detected significant ($p<0.05$) concentration change over time in phenanthrene, benz(*a*)anthracene, chrysene / triphenylene and benz(*g,h,i*)perylene, and marginally significant ($0.05<p<0.1$) concentration change over time in fluorene, anthracene, fluoranthene and pyrene. Variability in congener concentrations may have been due to contaminant cycling, the presence of residues prior to the initiation of the study, or imprecisions associated with frog injection. Dibenz(a,h)anthracene was the most persistent of the PAHs analyzed.

Concentrations of naphthalene, acenaphthene, acenaphthylene, phenanthrene, anthracene, benz(*k*)fluoranthene, benz(*a*)pyrene and benz(*g,h,i*)perylene fell below analytical detection limits (100 ng/g wet weight) in some of the frogs within the experimental time frame. These values were not used in linear regression analyses. Reductions in concentrations of naphthalene, acenaphthene, acenaphthalene, phenanthrene and anthracene in some of the frogs was most likely due to a combination of passive, hydrophobicity-based elimination based on their relatively low log K_{ow}s, as well as metabolism, since low molecular weight PAHs tend to be preferentially biodegraded (Shuttleworth and Cerniglia 1995). Naphthalene metabolites have been detected in the annelid *Neanthes arenaceodentata*, spider crab (*Maia squinado*), blue crab (*Callinectus sapidus*), spot shrimp (*Pandalus platyceros*), copepods (*Calanus spp.*), various amphipods, coho salmon (*Oncorhynchus kisutch*), and mudsucker (*Gillichthys mirabilis*) (reviewed in Neff 1979), as well as in salmonid and pleuronectid fish (Varanasi et al. 1989a). Phenanthrene was metabolized by brown bullheads (*Ameiurus nebulosus*) (Maccubbin et al. 1988). Anthracene was metabolized by the amphipod *Hyalella azteca* (Landrum and Scavia 1983), and by bluegill sunfish (*Lepomis macrochirus*) (Spacie et al. 1983).

However, the rapid elimination of benz(*k*)fluoranthene, benz(*a*)pyrene and benz(*g,h,i*)perylene, which have much higher log K_{ow}s (6.0, 6.04 and 6.5, respectively), are probably the result of metabolism. Varanasi and Gmur (1981) showed a much higher proportion of benz(*a*)pyrene metabolites compared to naphthalene metabolites in English sole (*Parphrys vetulus*) exposed to PAH-contaminated sediments. The polychaete worms *Nereis diversicolor* and *Scolecolepides viridis* were able to metabolize benz(*a*)pyrene extensively (Driscoll and McElroy 1996). The midge *Chironomus riparius* was able to transform 50% of accumulated benz(*a*)pyrene into polar metabolites in the matter of an hour (Leversee et al. 1982). The terrestrial isopod *Porcellio scaber* was able to quickly eliminate benz(*a*)pyrene, possibly due to metabolism (van Brummelen and van Straalen 1996). Bluegill sunfish (*Lepomis macrochirus*) were able to rapidly metabolize benz(*a*)pyrene (Spacie et al. 1983). Sea bass (*Dicentrarchus labrax*) were also able to metabolize benz(*a*)pyrene (Lemaire et al. 1992). Benz(*a*)pyrene metabolites have been detected in snails (*Physa spp.*), blue crab (*Callinectes sapidus*), copepods (*Calanus spp.*), the water flea (*Daphnia magna*), various amphipods, mosquito larvae (*Culex pipiens*), lake trout, mosquito fish (*Gambusia affinis*), mudsucker (*Gillichthys mirabilis*) and American toad embryos (*Bufo americanus*) (reviewed in Neff 1979). The amphibian *Pleurodeles waltl* was shown to metabolize benz(a)pyrene, and that clastogenic effects were related to the presence of benz(a)pyrene metabolites (Marty et al. 1989).

The rates of PAH biodegradation are variable and dependent on PAH structure and the metabolic capability of the organism (Shuttleworth and Cerniglia 1995). Generally, the lower molecular weight PAHs (3 rings or less) are more prone to biodegradation than PAHs that contain 4 or more rings (Shuttleworth and Cerniglia 1995). In fish, the route of PAH excretion is dependent on molecular weight: low molecular weight PAHs (benzene, naphthalene) tend to be eliminated by the gills, mucus and skin, and higher molecular weight PAHs need to be metabolized prior to excretion (Varanasi et al. 1989a). Also, certain molecular properties of individual PAHs may cause the cytochrome P450-based oxidation to take place in a region of the molecule that augments the metabolite's carcinogenicity. Studies have shown that the stearically hindered, angular regions of PAHs, such as that between the 4- and 5-positions of PHE

(known as the "bay region", Figure 12) are susceptible to oxidation, and these oxidation products (see Figure 6) are mutagenic and carcinogenic (Nordqvist et al. 1980).

The biological half-lives of various PAHs have been determined for a variety of organisms. BHL ranged form 0.02 (naphthalene) to 0.2 days (benz(*a*)anthracene) in the water flea (*Daphnia pulex*) (Southworth et al. 1978); 0.19 (phenanthrene) to 0.8 days (benz(*a*)pyrene) in mayflies (*Hexagenia limbata*) (Stehly et al. 1990); 1.57 (anthracene)to 2.17 days (benz(*a*)pyrene) in the aquatic oligochaete *Stylodrilus heringianus* (Frank et al. 1986); 3.9 (benz(*b*)fluoranthene) to 8 days (benz(*a*)pyrene) in the clam *Mercenaria mercenaria* (Bender et al. 1988); 11.9 (benz(*k*)fluoranthene) to 29.8 days (fluoranthene) in the mussel *Mytilus edulis* (Pruell et al. 1986); 3.4 (anthracene) to 26 days (fluoranthene) in the oyster *Crassotrea virginica* (Sericano et al. 1996; Bender et al. 1988); 0.71 (anthracene) to 2.79 days (benz(*a*)pyrene) in the bluegill sunfish (*Lepomis macrochirus*) (Spacie et al. 1983); 2.63 (anthracene) to 5.78 days (benz(*a*)pyrene) in zebrafish (Djomo et al. 1996); 1 (acenaphthylene) to 4 days (9-methyl anthracene) in rainbow trout (Niimi and Dookhran 1989); the BHL of anthracene was 1.9 days in the scud *Pontporeia hoyi* (Landrum 1982). The BHL (determined from statistically significant elimination rates) of PAHs in the northern leopard frogs used in this study range from 9.9 days (benz(a)anthracene) to 25.7 days (fluoranthene) and are within an order of magnitude of those found in the oyster and mussel, an order of magnitude slower than rainbow trout, bluegill sunfish, zebra fish, clam, oligochaete and the scud, and a few orders of magnitude slower than mayflies and water fleas. The comparability of these BHL, however, is limited due to the varying experimental conditions under which these studies were conducted.

The PAH elimination rates determined in this study do not show a significant linear correlation with log K_{ow}, meaning that PAH hydrophobicity does not seem to regulate elimination rate. When the statistically significant PAH elimination rates were plotted against hydrophobicity, a non-significant (p=0.1038) positive linear relationship was produced. Scatterplots of the elimination rates of other organisms against log K_{ow} show a variety of trends (Appendix 2). In the oyster, one study shows that elimination rates decline with increasing hydrophobicity (Bender et al. 1988), yet another shows no apparent relationship between elimination rate and hydrophobicity

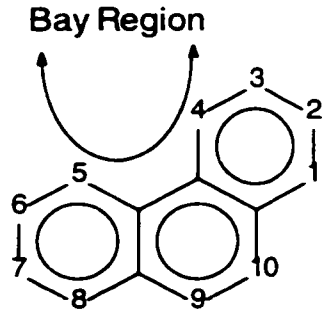


Figure 12. "Bay region" of phenanthrene.

(Sericano et al. 1996) (Appendix 2a). In the mussel, elimination rates seem to slightly increase with increasing hydrophobicity (Pruell et al. 1986) (Appendix 2b). In the clam (*Mercenaria mercenaria*), there does not appear to be any relationship between elimination rate and hydrophobicity (Bender et al. 1988) (Appendix 2c). In the oligochaete, PAH elimination rates decline with increasing log K_{ow} (Frank et al. 1986) (Appendix 2d). In the water flea, elimination rates also declined with increasing hydrophobicity (Southwort et al. 1978) (Appendix 2e). In zebra fish, elimination rates were related to hydrophobicity (Djomo et al. 1996) (Appendix 2f). In rainbow trout, elimination rates show a somewhat declining trend as hydrophobicity increases (Niimi and Dookhran 1989) (Appendix 2g). Linear regression analyses mostly show that PAH elimination rates are not related to hydrophobicity, except in the water flea (p=0.0079), the oysters in the study by Bender et al. (1988) (p=0.0037) and marginally in the oligochaete (p=0.0669) (Table 12). The literature referenced above indicates that the relationship between PAH elimination rates and hydrophobicity seems to be species-specific. The comparability of these relationships, however, is limited due to the varying experimental conditions under which these studies were conducted.

Elimination by passive diffusion, which is controlled by hydrophobicity, will yield a minimum elimination rate value; any biotransformation activity will only increase that rate of elimination (Meador et al. 1995). Where metabolism is substantial, the pattern of a decreasing rate of elimination with increasing hydrophobicity may be masked (Meador et al. 1995). In this study, the BHL of benz(*g,h,i*)perylene (10.5 days) is much less than the BHL of some of the other PAHs analysed, despite its relatively high log K_{ow}. In a study conducted by Schnell and others (1980), small differences in biological half life were observed between low and high molecular weight PAHs in salmon (*Oncorhynchus kisutch*) as hydrophobicity increased, due to the fast diffusion of the low molecular weight PAHs being balanced by the fast metabolism of the high molecular weight PAHs. In that same study, there was a two order of magnitude range in the affinity of monooxygenase for different PAH substrates, with benz(*a*)pyrene having a turnover rate 100 times that of naphthalene. To complicate matters, the length of exposure may affect the elimination of PAHs, especially the more hydrophobic ones. Short-term exposures may not allow the more hydrophobic PAHs to partition into and

Table 12. PAH elimination rates vs. log K_{ow} relationships from this and other studies. Rainbow trout (Niimi and Dookhran 1989); oyster 1 (Sericano et al. 1996); oyster 2 (Bender et al. 1988); mussel (Pruell et al. 1986); clam (Bender et al. 1988); oligochaete (Frank et al. 1986); zebra fish (Djomo et al. 1996); water flea (Southwort et al. 1978); northern leopard frog (this study). Asterisks indicate significance at the $p<0.05$ level.

Organism	Rainbow Trout	Oyster 1	Oyster 2	Mussel	Clam	Oligo-chaete	Zebra Fish	Water Flea	Northern Leopard Frog
Slope	-0.148	0.018	-0.115	0.010	-0.070	-0.069	-0.069	-14.8	0.012
Standard Error (slope)	0.104	0.015	0.019	0.008	0.059	0.019	0.041	3.01	0.006
Intercept	1.151	-0.015	0.713	-0.018	0.555	0.747	0.523	84.4	-0.017
Standard Error (intercept)	0.546	0.086	0.103	0.048	0.340	0.097	0.209	14.5	0.033
Correlation Coefficient	0.403	0.257	0.902	0.211	0.257	0.871	0.584	0.859	0.380
F value	2.02	1.38	36.84	1.6	1.38	13.46	2.8	24.29	3.67
Probability	0.25	0.3046	0.0037	0.2527	0.3045	0.0669	0.2361	0.0079*	0.1038

become sequestered by poorly-perfused, lipid-rich tissues, making them more labile and resulting in a relatively fast elimination rate (Meador et al. 1995).

The lack of a relationship between PAH elimination rates and log K_{ow} suggests that other routes of elimination are more critical than passive diffusion. Because adult anurans may not appreciably eliminate hydrophobic compounds via passive diffusion into their surroundings, elimination must occur by other routes. Strongly hydrophobic compounds undergo very little direct excretion in bile or urine, which leaves metabolism as the primary mechanism of the elimination of such compounds (Walker 1980; Walker 1987). In most terrestrial vertebrates, the metabolism of hydrophobic organic compounds occurs in the liver (Walker 1987). With this in mind, a two-compartment model may more accurately describe the elimination kinetics of such compounds from adult amphibians.

In this model, as proposed by Walker (1987), the organism consists of two compartments: the liver, and peripheral tissues. The liver obtains hydrophobic compounds from the digestive tract and by recirculation from peripheral tissues. Hydrophobic compounds are lost from the liver via metabolism and recirculation to peripheral tissues. In the adult amphibian, the uptake of hydrophobic contaminants may occur through dietary exposure, respiration, or transdermally. Encorporating Walker's (1987) model, contaminants obtained through dietary exposure could go directly to the liver or diffuse into peripheral tissues, whereas contaminants obtained through respiration or transdermally will diffuse into peripheral tissue. Contaminants in peripheral tissue may eventually arrive at the liver through recirculation, or be eliminated (depending on volatility) through respiration or diffusion through the permeable epidermis. Once these contaminants arrive at the liver, they may be metabolized and excreted, or redistributed back into peripheral tissues (Figure 13).

Overall, the sampling time frame used for measuring the elimination of PAHs may have been inappropriate. More measurements of PAH levels should have been taken near the beginning of the elimination period in order to better capture changes in PAH concentrations over time, especially with the lower molecular weight PAHs that were quickly eliminated. From a review of the literature, PAH metabolism appears to be

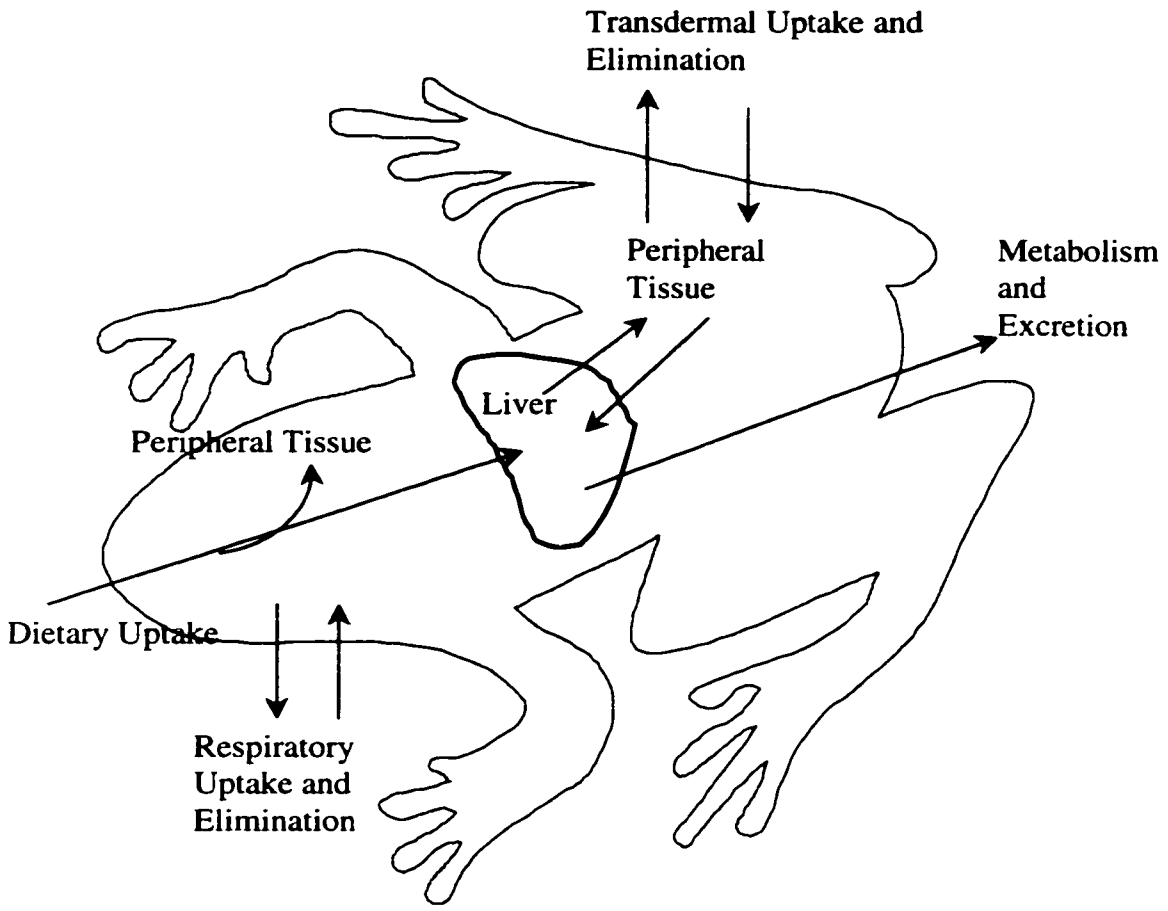


Figure 13. Proposed 2-compaartment model for adult amphibians. The compartments consist of the liver and peripheral tissues. Contaminants are introduced to the liver through dietary uptake or from peripheral tissues. Contaminants are eliminated from the liver through metabolism and excretion, or redistribution to peripheral tissue.

dependent on the PAH congener, as well as the species-specific ability to metabolize PAH congeners. In this study, the presence of detectable amounts of particular PAH congeners in some replicate frogs and not in others suggests that PAH congener metabolism may also depend on an individual frog's biotransformation ability, which could be a reflection of the frog's state of MFO enzyme activity.

In general, most of the PAHs were not as persistent as PCBs in the frogs used in this study (except for dibenz(*a,h*)anthracene). As with PCBs, PAHs that are persistent in a particular organism may pose a narcotic threat. Petersen and Kristensen (1998) suggestthat because PAH and PCB biotransformation was insignificant in egg and larval stages of fish, body burdens of these pollutants would be high in these early life stages. However, a review of the literature suggests that biomagnification of PAHs through trophic transfer will not increase the threat of narcosis because of the general increase in PAH biotransformation capabilities with increasing trophic level, and low dietary absorption efficiencies. However, the reduced threat of narcosis through PAH biotransformation is exchanged for the increased threat of genotoxicity resulting from the production of genotoxic PAH metabolites. Tanacredi and Cardenas (1991) caution that human consumption of bivalve molluscs chronically exposed to PAHs may lead to long-term health risks associated with the generation of genotoxic PAH metabolites. Uptake of PAHs from food sources appears to be an important route for filter feeders, but not in fish (NRC 1983). Niimi and Dookhran (1989) found that PAHs were not absorbed by rainbow trout through dietary exposure because of poor absorption efficiencies and rapid elimination rates. Clements et al. (1994) found that although midges (*Chironomus riparius*) mobilized PAHs from contaminated sediments, their predator (bluegill sunfish, *Lepomis machrochirus*) did not accumulate PAHs, and PAH metabolites were detected in the fish tissue. In a seston – blue mussel (*Mytilus edulis*) – eider duck (*Somateria mollissima*) food chain, the total concentrations of seven mutagenic PAHs decreased with increasing trophic level due to biotransformation (Broman et al. 1990). Porte and Albaigés (1993) found that in the western Mediterranean, PCBs are magnified with increases in trophic level, whereas PAHs are increasingly metabolized.

Adult amphibians may be inappropriate biomonitors of environmental concentrations of some PAHs. Metabolism is the principal confounding factor in

assessing exposure to certain PAH congeners: measurement of parent PAHs in an organism capable of metabolizing them will give only partial information about exposure and total PAH body burden (Meador et al. 1995). Additionally, species which are able to extensively metabolize certain PAH congeners should not be used as biomonitors of those congeners, as it would be expensive to monitor for the metabolites in addition to parent compounds (James 1989). The kinetics of individual PAHs in an organism may change over time due to enzyme induction, irreversible binding to cellular structures and macromolecules, and changes in the proportion and distribution of the parent compound and its metabolites (Spacie et al. 1983).

2) PCB vs. PAH Elimination Kinetics

In the frogs used in this study, most of the PAHs had faster elimination kinetics than all the PCBs. Metabolism may explain the difference in elimination rates between PCBs and PAHs of similar hydroophobicity in this study. PCBs 105 and 110, with respective log K_{ow}s of 6.65 and 6.48, showed no significant elimination, while benz(*g,h,i*)perylene, with a log K_{ow} of 6.5, had an elimination rate of 0.066 d^{-1}. In the amphipod *Pontoporeia hoyi*, the elimination rate of benz(*a*)anthracene was 0.0022 h^{-1}, while for PCB 52 (which has a similar log K_{ow}) elimination was not detected (Landrum 1988). In separate studies investigating the elimination of PAHs (Djomo et al 1996) and PCBs (Fox et al. 1994) from zebra fish (*B. rerio*), the BHL of pyrene (log K_{ow} 5.18) was 4.81 days, which was comparable to the BHL of 2.4 days obtained for PCB 18 (log K_{ow} 5.24); however, the BHL of 5.78 days obtained for benz(*a*)pyrene (log K_{ow} 6.04) was much less than the BHL of 28.2 days obtained for PCB 70 (log K_{ow} 6.2). In separate studies investigating the elimination of PAHs (Niimi and Dookhran 1989) and PCBs (Niimi and Oliver 1983) from rainbow trout (*O. mykiss*), the BHL of 2- and 9-methylanthracene (2 and 4 days, with log K_{ow}s of 5.15 and 5.07, respectively) was comparable to the BHL of 5 days for PCB 11 (log K_{ow} 5.28), but much less than BHL of PCB 9 (85 days, log K_{ow} 5.06). The greater relative persistence of the mono *ortho*-substituted PCB 9 could be due to metabolism of PCB 11 (non *ortho*-substituted) and the PAHs. In the same studies, the BHL of triphenylene (2 days, log K_{ow} 5.49) was much

less than the BHL of PCB 31 (196 days, log K_{ow} 5.67), and the BHL of perylene (2 days, log K_{ow} 6.25) was much less than the BHL of PCB 72 (890 days, log K_{ow} 6.26).

Tulp and Hutzinger (1978) assert that metabolism is the only factor that can account for the fact that two compounds with minimal differences in hydrophobicity and steric configuration can behave totally differently with respect to bioaccumulation. Indirect evidence of this is shown in a study conducted by Ma et al. (1998) where elimination rate constants for several PAHs were compared with available data on elimination rate constants for chlorobenzenes of corresponding K_{ow} range. For chlorobenzenes, elimination rates tend to decrease with increasing K_{ow}, whereas for the PAHs, the elimination rate failed to show a strong decline over a similar range of increasing K_{ow}. Oliver and Niimi (1985) found that for some high molecular weight organochlorinated and brominated compounds and compounds metabolized by rainbow trout, bioconcentration factors did not correlate well with K_{ow}. Hydrophobic compounds that are able to be biotransformed in fish (such as PAHs) generally show less bioaccumulation than PCBs, which are more persistent (Van der Oost et al. 1991). In *Chironomus tentans* larvae, metabolism could be responsible for the lower bioaccumulation factors of the PAHs compared to the PCBs (Wood et al. 1997). In contrast, the slope of the statistically non-significant concentration-time relationship of dibenz(*a,h*)anthracene determined in this study is within the same range as that of the PCBs of comparable log K_{ow}. Because of the similar behaviour of this particular PAH and PCBs of corresponding log K_{ow}, dibenz(*a,h*)anthracene elimination may be controlled by the same processes that control PCB elimination. Comparison of the bioaccumulation of persistent compounds (such as PCBs) to that of PAHs may give a good indication of whether or not PAHs are being metabolized, even when it is not possible to measure metabolites directly (McElroy et al. 1989).

A review of the literature reveals that concentrations of PAHs and PCBs in environmental matrices (e.g. sediment) and biota depend on the proximity to urbanized areas and point sources of these compounds, the chemical composition of the pollution, as well as the metabolic capabilities of the organisms. Average PCB levels were an order of magnitude higher than average PAH levels in mussels (*Mytilus galloprovincialis*), red mullets (*Mullus barbatus* and *M. surmuletus*), crabs (*Macropipus spp.*) and tuna

(*Thunnus thynnus*) collected from non-urbanized areas of the Mediterranean sea; levels of both PCBs and PAHs in these organisms were ten-fold higher in close proximity to large urban areas than in less populated areas (Porte and Albaigés 1993). PCB levels were much less than PAH levels in sediments collected in close proximity to aluminum processing and automobile manufacturing facilities (Wood et al. 1997). Similarly, shellfish (*Macomona liliana* and *Austrovenus stutchburyi*) collected from Manukau Harbour in New Zealand had higher PAH body burdens than PCB body burdens (Hickey et al. 1995). In the heavily polluted Trenton Channel of the Detroit River, brown bullheads (*Ameiurus nebulosus*) had PCB levels of several hundred ng/g dorsal muscle tissue (lipid-corrected) and non-detectable levels of PAHs, even though sediment concentrations of PCBs were only somewhat higher than that of PAHs; the low levels of PAHs were attributable to the rapid metabolism of PAHs (Leadley et al. 1998). In the Rhine and Meuse rivers, sediment PAH concentrations ranged in the thousands of ng/g dry weight, while PCB levels were largely not detected; however, in zebra mussels (*D. polymorpha*) and eel (*Anguilla anguilla*) collected from those rivers, PAH levels were similar to PCB levels in zebra mussels, while PAHs were not detected in eel tissue (Hendriks et al. 1998). Interestingly, both of these organisms had detectable levels of PCB congeners that were not detected in the sediment.

Generally, PCBs and PAHs behaved differently with respect to biological persistence in the frogs used in this study. The rapid elimination of the PAHs relative to the PCBs, as well as the poor relationship between PAH elimination rate and log K_{ow}, implies that some of the PAH congeners were metabolized by the frogs used in this study.

Summary and Conclusions

PCBs were biologically persistent in the adult frogs used in this study. Because of this, adult amphibians may be excellent biomonitors of environmental PCB levels but they should not be used to track temporal PCB fluctuations because of their slow PCB elimination kinetics. Further studies of substantially longer time frame, as well as the development of kinetic models that reflect the unique physiologies of adult amphibians, should be conducted to determine if log K_{ow} could be used to estimate PCB persistence in these organisms.

Elimination kinetics were generally faster for the PAHs than the PCBs. The lack of a relationship between hydrophobicity and PAH elimination rates suggests that other factors, such as metabolism, may be more important in their elimination than passive diffusion. In light of this, a two compartment kinetic model may be more appropriate to describe PAH elimination in these organisms. If the frogs used in this study are representative of other adult amphibians, they should not be used as biomonitors for some PAHs because of the confounding factors associated with potential PAH metabolism.

The faster elimination kinetics of PAHs compared with PCB congeners of comparable log K_{ow} supports that PAH metabolism is taking place. Comparison of the behaviour of PAHs to the biologically persistent PCBs indirectly indicates that several PAH congeners may have been metabolized in this study.

The persistence of PCBs in the frogs used in this study suggests that chronic exposure to PCBs may result in their accumulation in sufficient quantities to cause narcosis and affect reproductive success. In addition, the ability of some PCB congeners to induce and increase oxidative enzyme (MFO) level leads to the increased metabolism of other hydrophobic organic contaminants, such as PAHs, potentially resulting in the production of genotoxic metabolites. Chemically induced genetic abnormalities could become incorporated in an amphibian community because of their limited dispersal abilities, site fidelity and metapopulation-like dynamics. Because of the biological persistence of PCBs and the global ubiquity and genotoxic potential of PAHs, these classes of environmental contaminants may decrease the fitness of amphibian communities.

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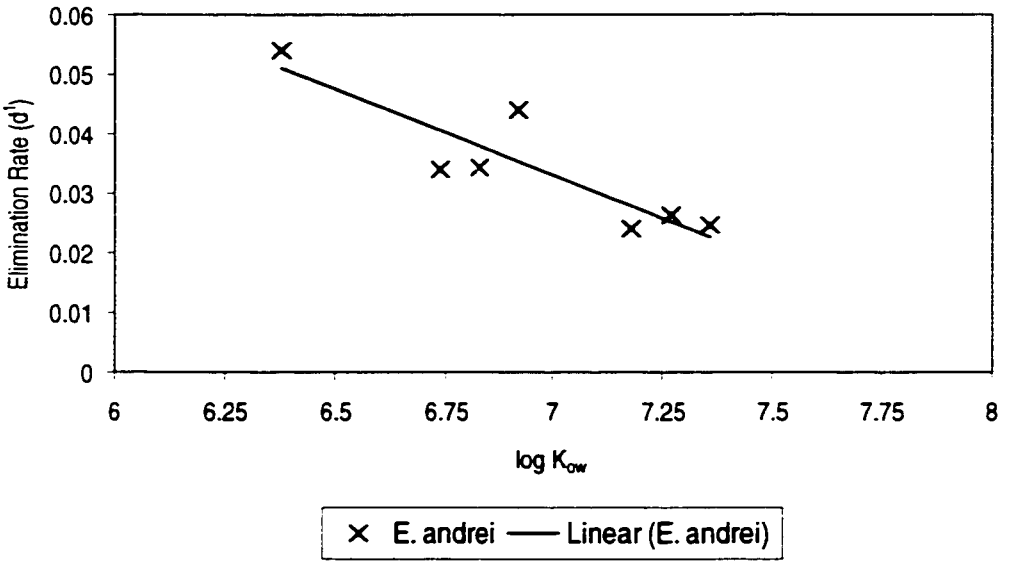
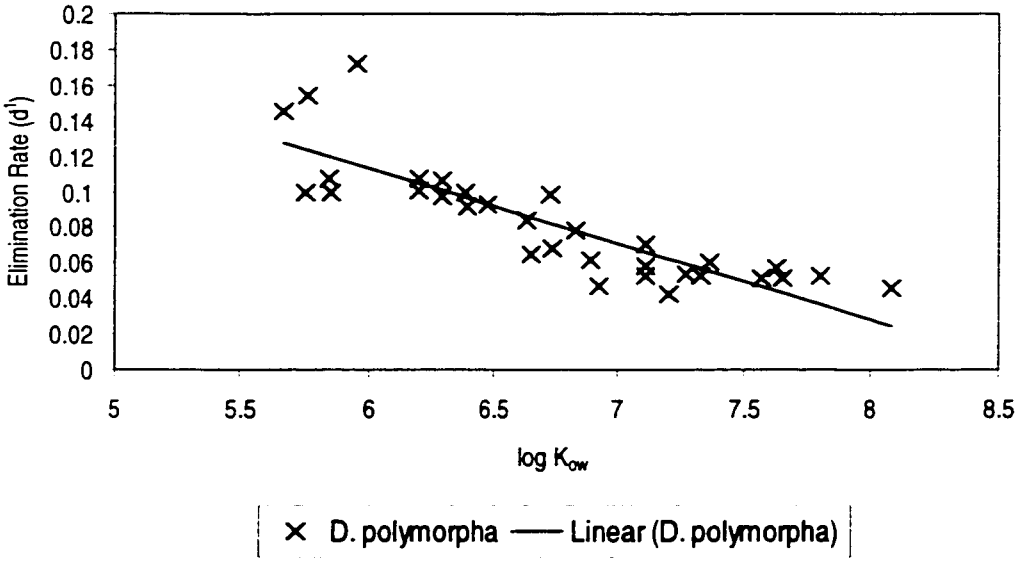
Appendices

Appendix 1: Elimination – log Kow scatterplots of PCBs from other studies. Points represent elimination rates taken from the following organisms and studies: a) earthworms (Belfroid et al. 1995); b) zebra mussels (Morrison et al. 1995); c) green-lipped mussels (Tanabe et al. 1987); zebrafish (Fox et al. 1994); e) adult rainbow trout (Coristine et al. 1996); f) juvenile rainbow trout (Fisk et al. 1998). Trendline represents the line of best fit (linear).

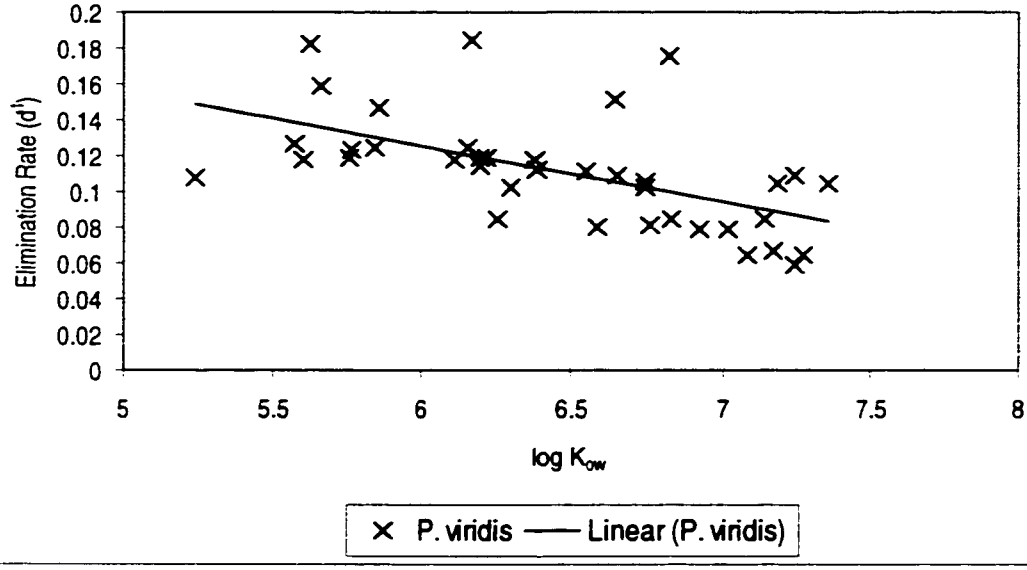
a) Elimination Rate - Hydrophobicity Relationship for PCBs in Earthworms (*Eisenia andrei*)



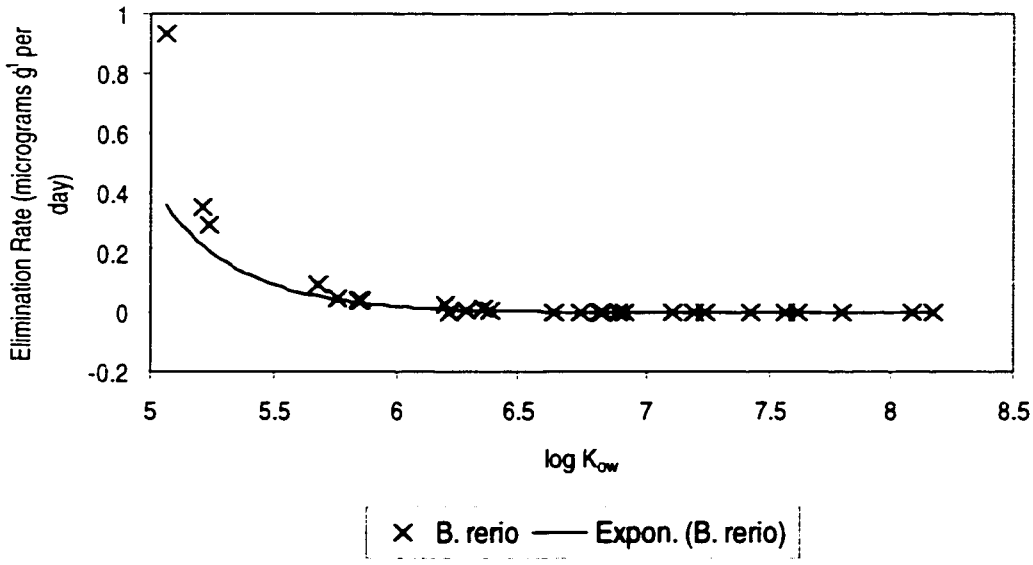
b) Elimination Rate - Hydrophobicity Relationship for PCBs in Zebra Mussels (*Dreissena polymorpha*)



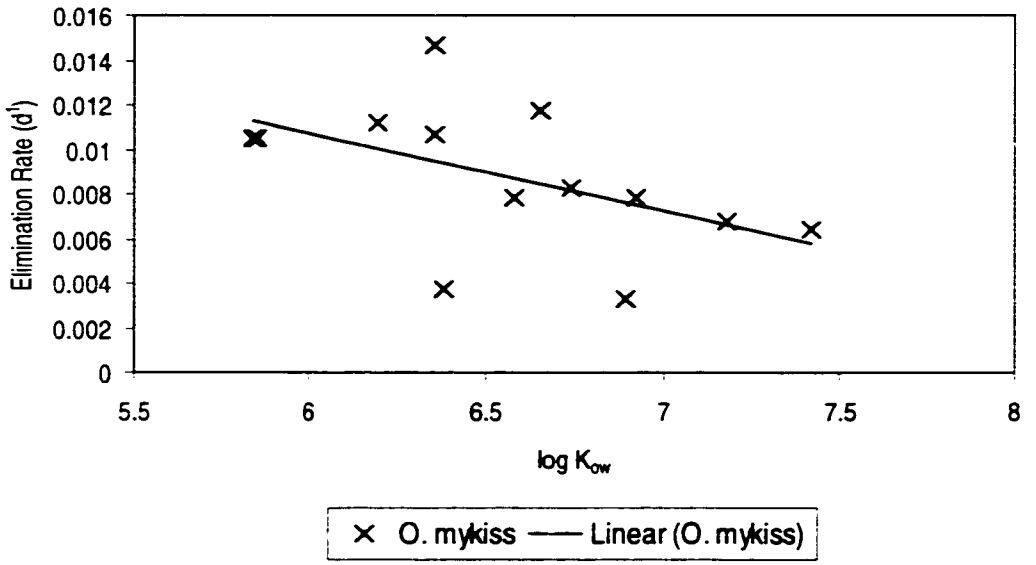
c) Elimination Rate - Hydrophobicity Relationship for PCBs in Green-lipped Mussels (*Perna viridis*)



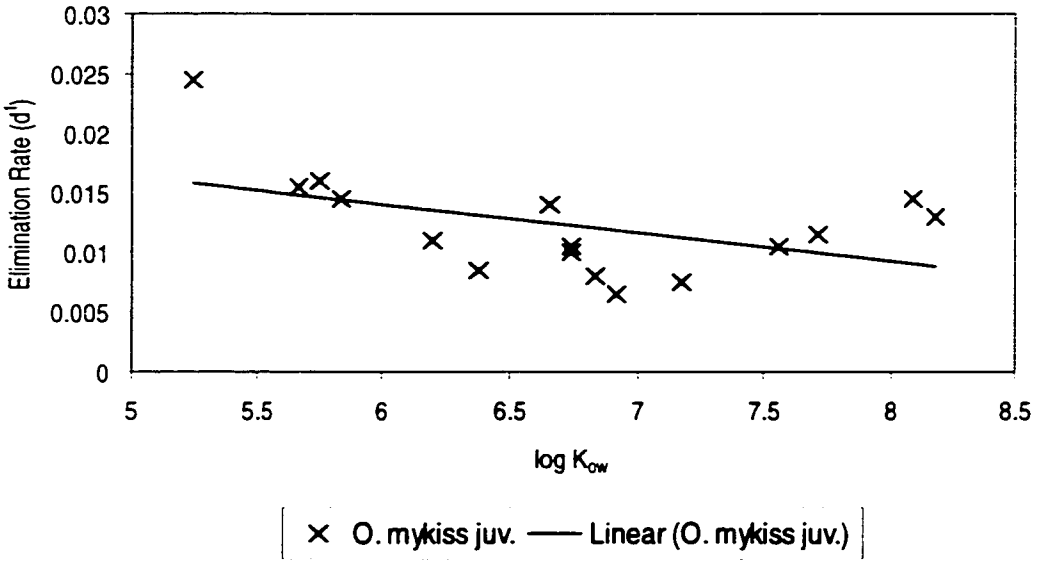
d) Elimination Rate - Hydrophobicity Relationship for PCBs in Zebra Fish (*Brachydanio rerio*)



e) Elimination Rate - Hydrophobicity Relationship for PCBs in Adult Rainbow Trout (*Oncorhynchus mykiss*)

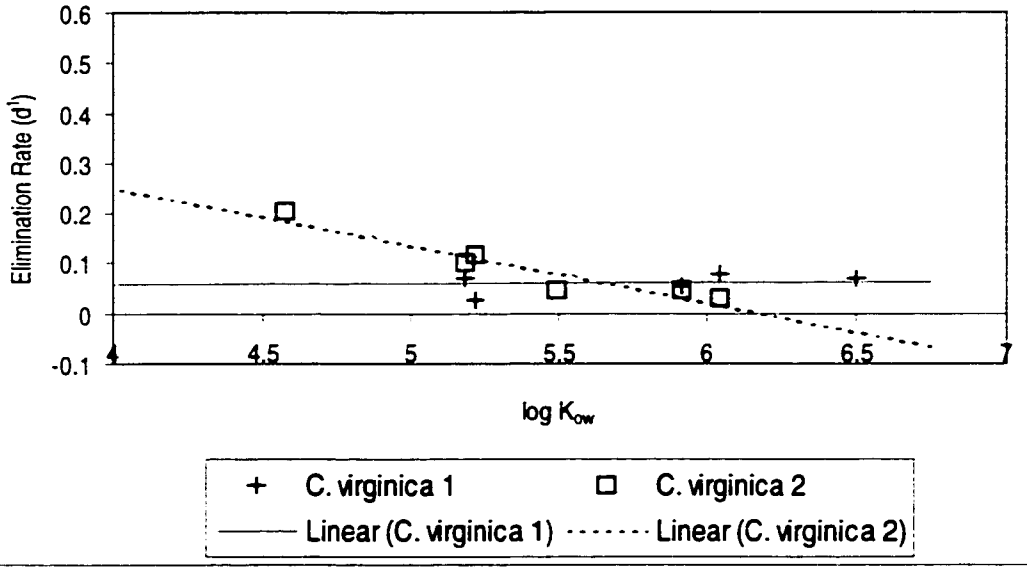


f) Elimination Rate - Hydrophobicity Relationship for PCBs in Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

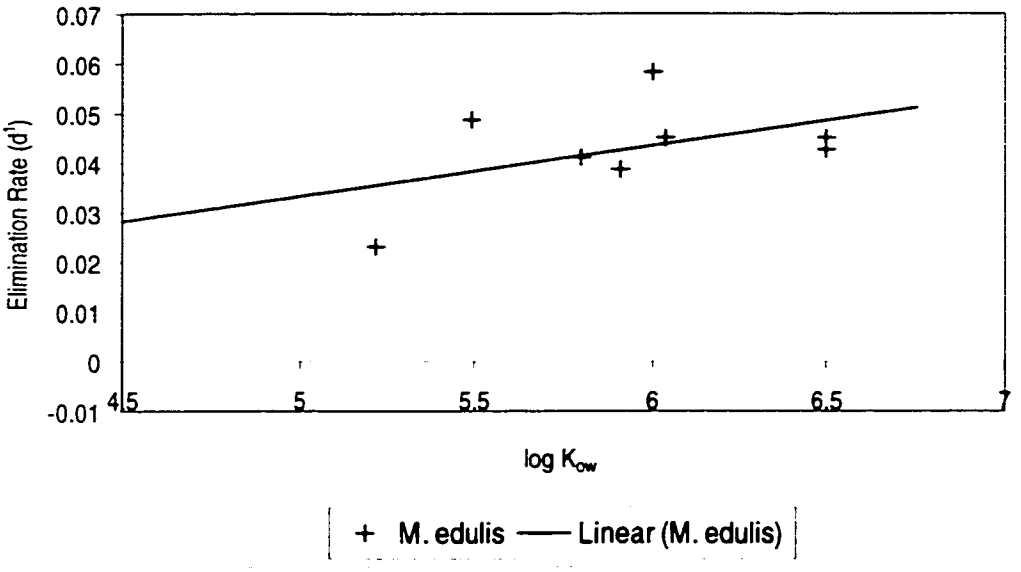


Appendix 2: Elimination – log K_{ow} scatterplots of PAHs from other studies. a) oyster 1 (Sericano et al. 1996) and oyster 2 (Bender et al. 1988); b) mussel (Pruell et al. 1986); c) clam (Bender et al. 1988); d) *Stylodrilus* (Frank et al. 1986); e) *Daphnia* (Southwort et al. 1978); f) zebra fish (Djomo et al. 1996); g) rainbow trout (Niimi and Dookhran 1989).

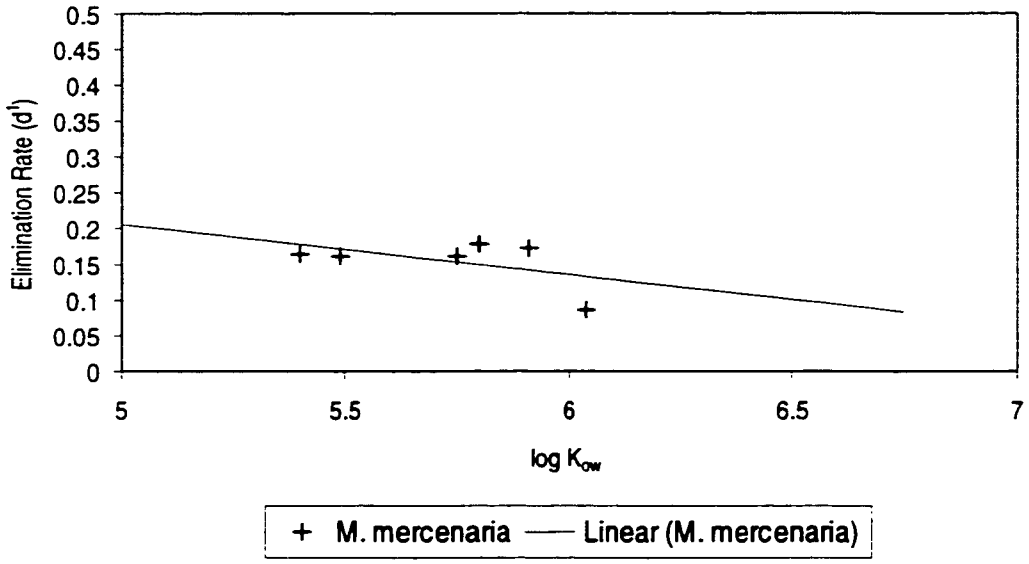
a) Elimination Rate - Hydrophobicity Relationship for PAHs in the Oyster (*Crassostrea virginica*)



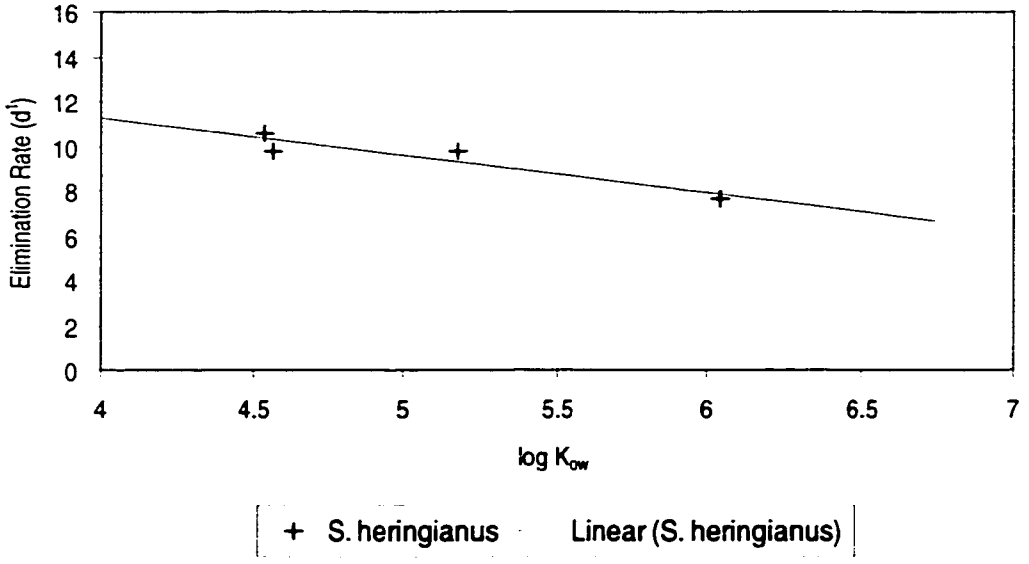
b) Elimination Rate - Hydrophobicity Relationship for PAHs in the Mussel (*Mytilus edulis*)



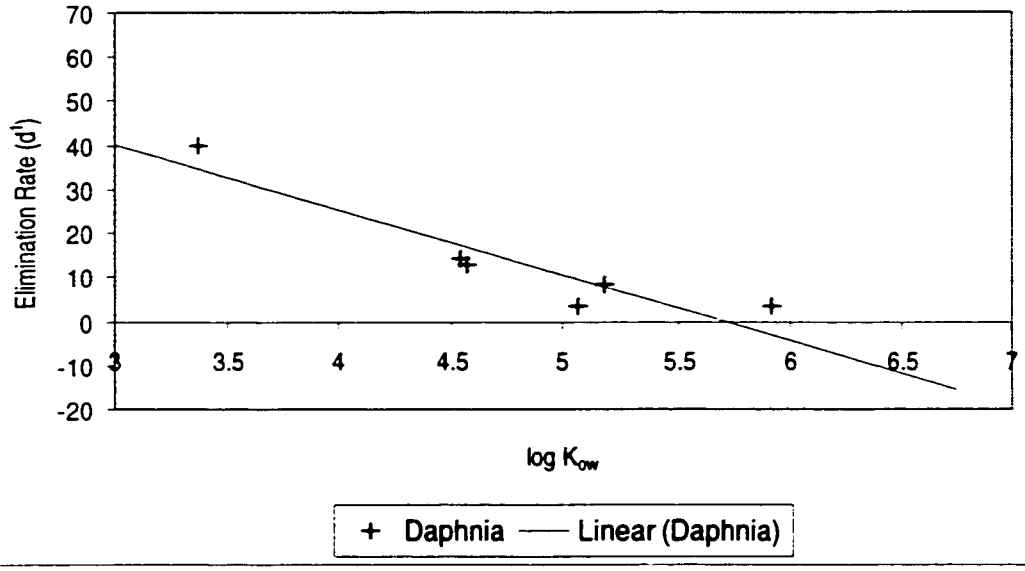
c) Elimination Rate - Hydrophobicity Relationship for PAHs in the Clam (*Mercenaria mercenaria*)



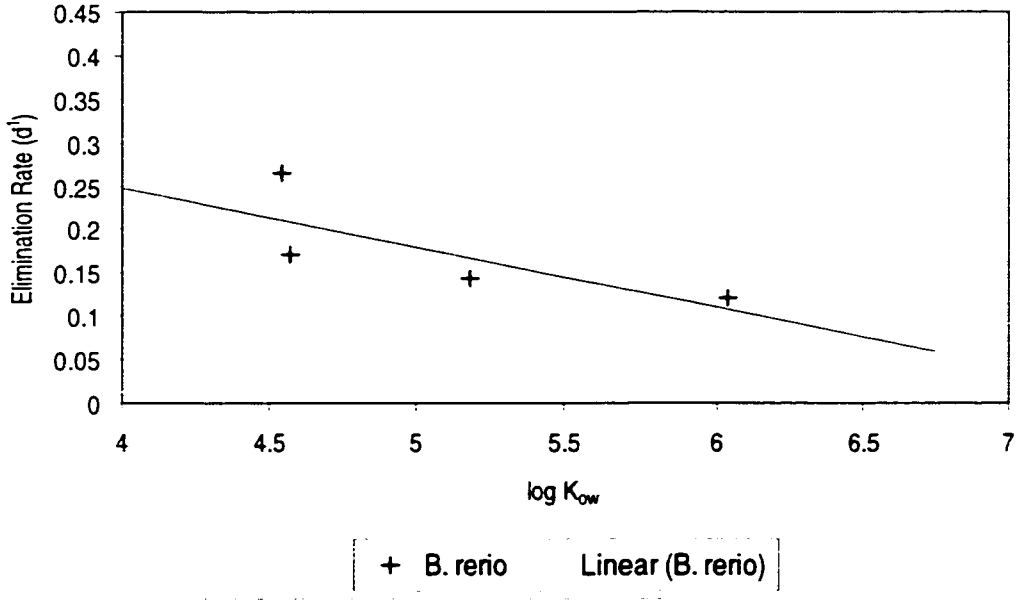
d) Elimination Rate - Hydrophobicity Relationship for PAHs in the Oligochaete (*Stylodrilus heringianus*)



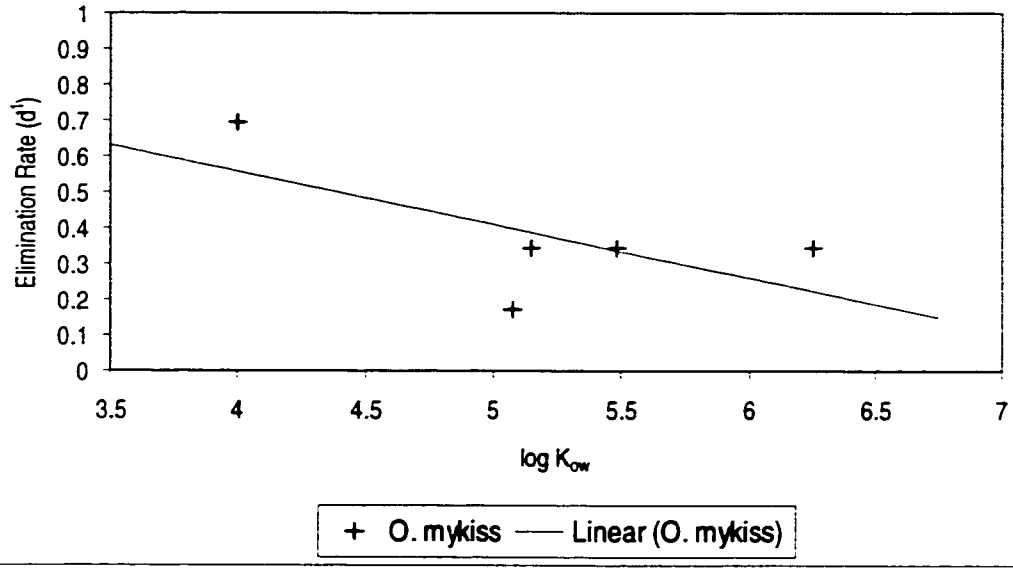
e) Elimination Rate - Hydrophobicity Relationship for PAHs in the Water Flea (*Daphnia pulex*)



f) Elimination Rate - Hydrophobicity Relationship for PAHs in Zebra Fish (*Brachydanio rerio*)



g) Elimination Rate - Hydrophobicity Relationship for PAHs in Rainbow Trout (*Oncorhynchus mykiss*)



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